THE INDUCED SYNTHESIS AND PROPERTIES OF BETA-GALACTOSIDASE OF *SACCHAROMYCES FRAGILIS*

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B.A., University of British Columbia, 1949
M.S., University of Illinois, 1952

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BACTERIOLOGY
IN THE GRADUATE COLLEGE OF THE
UNIVERSITY OF ILLINOIS, 1957

URBANA, ILLINOIS

UNIVERSITY OF ILLINOIS

THE GRADUATE COLLEGE

December 14, 1956

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Marion Helena Nyholm Gilmour

ENTITLED The Induced Synthesis and Properties of β-Galactosidase of Saccharomyces Fragilis

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Ph.D.

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† Required for doctor's degree but not for master's.

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ACKNOWLEDGEMENTS

The writer wishes to express her thanks to Dr. S. Spiegelman for his suggestion of the general problem, his helpful criticisms and discussions of it, and his continuing encouragement.

She is also indebted to Dr. Hugh S.A. Gilmour for his aid in discussions of kinetics and his criticisms during the preparation of this manuscript.

Much of the experimental work reported herein has been performed with the able and conscientious assistance of Miss Maria Bernardini.

I. INTRODUCTION

A. General Background

The synthesis of an enzyme by a cell can be classified either as inducible or constitutive according to whether it is synthesized by exposure of the cell to a specific chemical compound, i.e. inducible enzyme synthesis, or synthesized in the absence of a specific chemical compound, i.e. constitutive enzyme synthesis. The type of synthesis which will occur is determined by the cell's genetic constitution (1,2).

Induced enzyme formation has been shown to be a de novo protein synthesis which occurs by the simultaneous union of amino acids rather than by the assemblage of preformed blocks of peptide precursors (3,4,5,6,7). Presumably constitutive enzyme formation is also a de novo protein formation. A knowledge of the difference in mechanism between the two types of enzyme formation would be of value to an understanding of both gene action and protein synthesis. The first problem is to determine if the mechanisms of induced and constitutive enzyme formation are essentially identical, differing perhaps at one key point, or if the mechanisms are totally unlike. The primary objective of this study is to differentiate between the above two alternatives.

In studying this problem, two main lines of investigation have been taken: a comparison of the enzymes elaborated inducibly and constitutively, and a comparison of the kinetics of their synthesis. The kinetic study is based upon the suggestion that qualitatively similar kinetics are indicative that the same rate-limiting steps are involved in both types of enzyme formation.

The beta-galactosidase system in S. fragilis was chosen for this study. Because the organism synthesized measurable levels of beta-galactosidase activity constitutively and inducibly, the study of both types of enzymes could be performed against a genetically constant background. The pattern of induction and the properties of both the constitutive and induced enzymes were ill defined, and have therefore been characterized.

B. Scope of the Present Investigation

The studies to be presented focus attention on the following aspects of formation of beta-galactosidase activity in constitutive and induced cells of S. fragilis.

1. The properties and specificity patterns of the enzymes synthesized have been studied. It will be shown that both magnesium and potassium ions are required for enzyme activity, although previously one or the other had been reported an activator of the induced enzyme (8,9). Furthermore, both enzymes have been found to hydrolyze all beta-galactosides tested, and should therefore be termed beta-galactosidases.
2. The number of enzyme species having beta-galactosidase activity is unknown and has therefore been investigated. The data presented herein clearly show the presence of one stable enzyme in the induced cells and one stable enzyme in the constitutive cells of S. fragilis.
3. The specificity of the induction has been studied, and is compared to the specificity of the enzyme. The specificities of the beta-galactosidase and its enzyme forming system are shown to be the

same in S. fragilis and are different from the specificities of the beta-galactosidase system in E. coli (10).

4. The effect of inducer on the crypticity of the enzyme has been investigated. It is demonstrated that the crypticity factor always decreases with induction, the amount of decrease being dependent upon the substrate tested.
5. The properties of the induced and constitutive enzymes have been compared. By all the tests applied, the enzyme in constitutive cells is shown to be identical to that in induced cells.
6. The kinetics of the induced and constitutive enzyme synthesis are demonstrated to be qualitatively similar.
7. The effect of generation time (g) on the relative rate of enzyme formation (dE/dM where E is enzyme and M is mass), has been studied. It is shown that there is a dependence of dE/dM upon g during some phases of the growth cycle for both constitutive and induced enzyme formation.

C. Previous Investigations on the Lactase of S. fragilis

Capputto, Leloir, and Trucco (8) undertook a study of the purification and properties of the induced yeast lactase in 1947. Purification was accomplished by acetone and ammonium sulfate fractionations, the latter accompanied by large losses of enzyme activity. The enzyme was unstable, but could be stabilized at pH 6.8 in 0.6 M KCl or 0.1 M citrate buffer. On the basis of their investigations, the properties of the enzyme can be summarized as follows:

1. pH optimum 6.7-6.9,
2. activation by magnesium, potassium and manganese ions in that

order of effectiveness,

3. inactivation by cuprous and zinc ions, and
4. one equivalent of lactose is hydrolyzed to form one equivalent of galactose and one equivalent of glucose, by measurement of increase in reducing sugars.

Connors and his co-workers (9) have purified the induced enzyme 25-fold by ammonium sulfate and acetone fractionation, and have examined its properties. The enzyme was found to be unstable upon dialysis or storage. Its pH optimum was 6.2-7.0. Activation of enzyme activity occurred in the presence of magnesium,azide, arsenite, and potassium ion, whereas inactivation was observed in the presence of copper, mercury, and lead ions.

The nature of the reaction which is catalyzed in the presence of the enzyme has been the subject of many reports. As noted, Leloir et al. (8) found one mole of glucose and one mole of galactose per mole of lactose. However, when the enzyme has been employed in high concentrations, oligosaccharides composed of galactose or varying ratios of glucose to galactose have been found (11,12,13,14). Aronson (14) reports a glucose to galactose ratio of 1:1 in one oligosaccharide and a ratio of 1:2 in a second. In his experiments, xylose and glycerol could act as acceptors for the galactose moiety as well as glucose and galactose. In comparable experiments with the E. coli beta-galactosidase, three oligosaccharides were isolated; in two the glucose to galactose ratio was 1:1, the third was comprised solely of galactose. Since the latter two types of oligosaccharides were not hydrolyzed by the yeast lactase, it is unlikely that they have the same configuration as the oligosaccharides produced by the yeast enzyme. Thus, the yeast lactase and the E. coli beta-galactosidase appear to act both as hydrolytic and transference enzymes.

The rate of CO_2 evolution from lactose might *a priori* be expected to be the same as that from a mixture of the constituent hexoses if: a) the pathways of glucose and galactose metabolism are the same as those for the products of lactose breakdown by lactase, and b) the rates of permeability of the disaccharide and its constituent hexoses are similar. However, there are a number of reports (15,16,17,18,19) demonstrating that lactose grown *S. fragilis* utilizes lactose at a greater rate than the constituent hexoses singly or in mixtures. This fact has been interpreted to demonstrate the existence of a series of enzymes for the direct utilization of lactose. Intensive efforts to isolate such enzymes have been unsuccessful.

The lactase of *S. fragilis* has been shown to be an inducible enzyme by both Taylor (19) and Davies (20). However, no experiments have been performed to determine the specificity of either the induction or the enzyme.

II. MATERIALS AND METHODS

A. Description of Yeast Strains

Saccharomyces fragilis, strain Y 665, and strain FPR-1, a hybrid of Zygosaccharomyces dobzhanskii and S. fragilis (21) were obtained from the Northern Regional Laboratories. The two parent strains are heterothallic diploids. Both strains Y 665 and FPR-1 produce a low level of beta-galactosidase constitutively, and form higher levels of the enzyme under the influence of lactose. The hybrid is also inducible for maltase, synthesizing low levels of this enzyme constitutively.

In addition to these strains, Saccharomyces cerevisiae, strain LK_2G_{12}, and Torula monosa have been employed. The former can be induced for galactozymase activity, whereas the latter ferments neither galactose nor lactose. These strains were used in the assay systems described below.

B. Carbohydrates Employed

Carbohydrates employed in this investigation were either re-crystallized A. R. grade or were galactosides synthesized in the laboratory. Galactose, maltose, alpha-methyl glucoside and melibiose were purified of contaminating fermentable sugars by the method described by Spiegelman (22). Two or three re-crystallizations from 70 % alcohol were found adequate for purification. For mass induction experiments, 10 % aqueous solutions of galactose were purified of contaminating dextrose by fermentation with heavy suspensions of Torula monosa.

Methyl-beta-D-galactoside, phenyl-beta-D-galactoside, and

ortho-nitrophenyl-beta-D-galactoside were synthesized by the method of Seidman and Link (23). Methyl-alpha-D-galactoside, phenyl-alpha-D-galactoside, and phenyl-alpha-D-glucoside were synthesized according to the method of Montgomery, Richtmeyer, and Hudson (24). n-butyl-beta-D-galactoside, methyl-beta-D-thiogalactoside, and phenyl-beta-D-thiogalactoside were kindly supplied by Dr. J. Monod, Institut Pasteur.

C. Cell Growth

1. Measurement of cell growth

The density of cell suspensions was measured turbidimetrically with a Coleman Junior Spectrophotometer Model 6A which had been standardized with a didyium standard. The instrument was zeroed with a medium blank, and measurements taken at a wavelength of 440 millimicrons. For convenience in growth experiments, a set of 20 x 2.5 cm tubes were standardized, and turbidity readings made directly on the growth tube. The optical density values were converted into mgm dry weight of cell material by reference to the graph of O.D. vs dry weight shown in Figure 1.

2. Media employed

A complete medium of the following composition was used:

Difco bacto-peptone	5.0 gm
Difco yeast extract	2.5 gm
$(NH_4)_2SO_4$	2.0 gm
$CaCl_2$	0.25 gm
$MgSO_4$	0.25 gm
60 % sodium lactate	6.0 ml
Distilled water	1000 ml

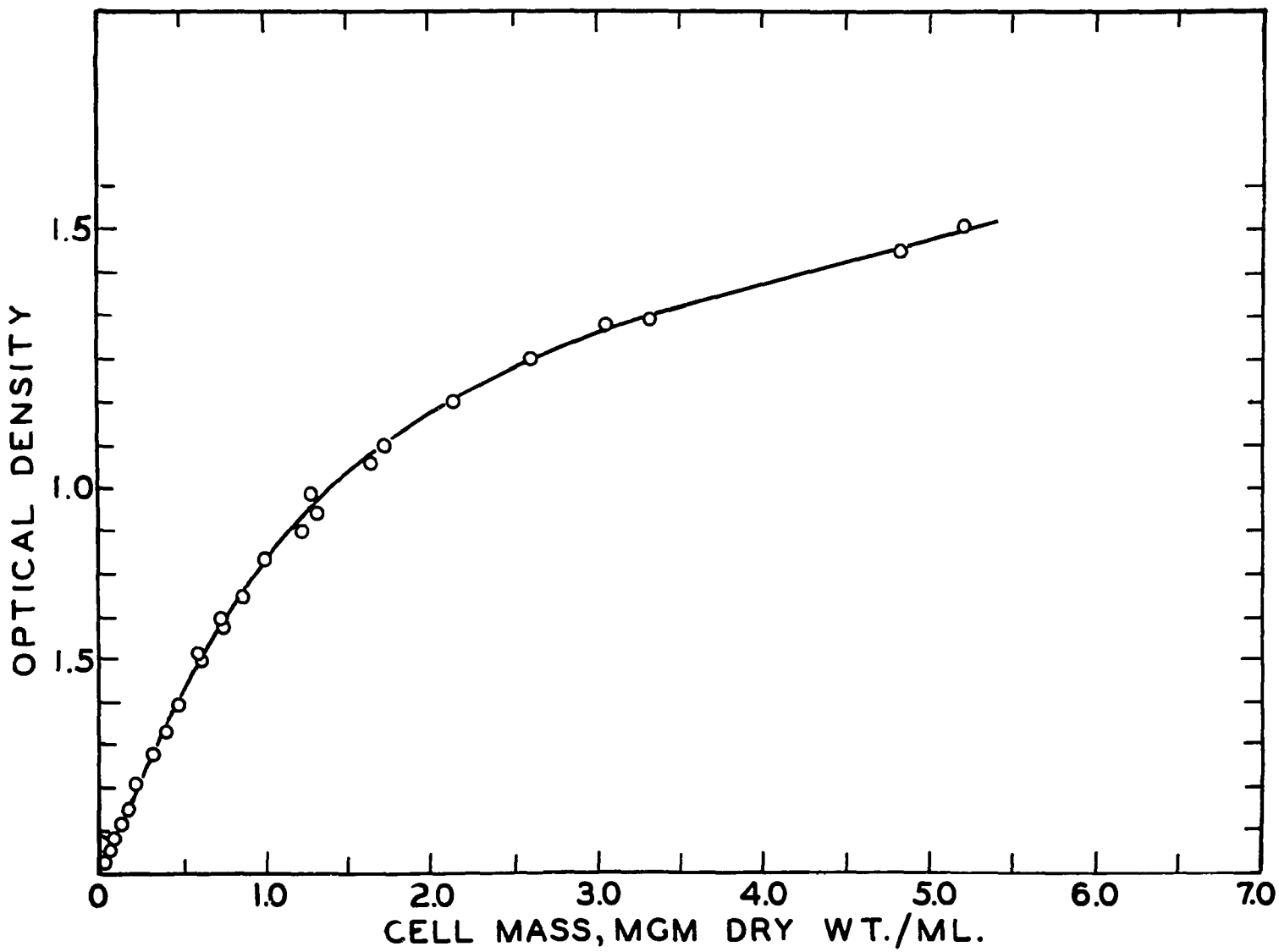


Fig. 1. Relation between optical density and dry weight.

The medium was autoclaved for 15 min at 15 lbs per sq in. Carbon sources of 1 % ethanol or 2 % dextrose final concentrations were added after sterilization.

For controlled growth and induction experiments, modifications of three synthetic media have been employed: Burkholder's medium (25), a variation of Burkholder's medium having a pH of 6.5, and Davies medium (20). As a result of preliminary experiments the buffering capacities of these media were increased by a factor of five. The carbon source was 1 % glycerol or 1 % ethanol.

The composition of the three media is given below.

a. Burkholder's media (modified).

	for pH 5	for pH 6.5
K_2HPO_4	0	2.42 gm
KH_2PO_4	7.5 gm	5.35 gm
$MgSO_4.7H_2O$	0.5 gm	0.5 gm
$CaCl_2.2H_2O$	0.33 gm	0.33 gm
$(NH_4)_2SO_4$	2.0 gm	2.0 gm
KI, 0.1 % aqueous solution	0.1 ml	0.1 ml
Distilled water	1000 ml	1000 ml
1 M NaOH solution	to pH 5	0

Trace elements, ppm final concentration

B	0.01	Cu	0.01
Mn	0.01	Mo	0.01
Zn	0.07	Fe	0.05

Vitamins, micrograms per liter final concentration

Thiamine HCl	200
Riboflavine	100

B_6	200
Niacin	200
Biotin (methyl ester)	0.2
Pantothenic acid (Ca)	200
Inositol	1000

Vitamins were sterilized by filtration and added to sterile medium. The medium was autoclaved 15 min at 15 lbs per sq in.

b. Davies medium (modified).

$NH_4H_2PO_4$	5.0 gm
$(NH_4)_2HPO_4$	5.0 gm
KH_2PO_4	10.0 gm
Trace elements	10.0 ml
Vitamins	10.0 ml
Distilled water	1000 ml

The medium was autoclaved 15 to 20 min at 15 lbs per sq in.

Trace element solution

$MgSO_4.7H_2O$	2.0 % w/v
NaCl	0.1 % w/v
$FeSO_4.7H_2O$	0.05 % w/v
$ZnSO_4.7H_2O$	0.05 % w/v
$MnSO_4.3H_2O$	0.05 % w/v
$CuSO_4.5H_2O$	0.005 % w/v
H_2SO_4, 0.1 N	1 % v/v

Vitamin solution, mg per 100 ml

Biotin	0.001
Ca pantothenate	0.3
Pyridoxine	3.3

Nicotinic acid	3.3
Inositol	3.3
Thiamine	3.3

Following sterilization by filtration through UF sintered glass filters, the trace element and vitamin solutions were stored at 5°C.

3. Cultures

Stock cultures were maintained on the complete medium supplemented with 2 % dextrose and agar. Transfers were made weekly.

15 ml of suitable medium in a 20 x 2.5 cm test tube were inoculated from the stock culture and incubated at 30°C with aeration for 20 hours. 0.5 ml of this culture served as inoculum for 75 ml of the same medium in a 750 ml erlenmeyer flask. The flask was agitated on a rotary shaker until an O.D. of 0.3 was attained; the length of incubation was dependent upon the medium. This culture was employed for inoculating experimental cultures. The inoculum (25 ml for unsupplemented synthetic medium, and 0.5 ml for complete medium) was added to 250 ml medium in a 2 liter flask, and the culture was incubated at 30°C on a shaker rotating at 150 to 165 rpm.

Growth tubes were aerated by placing them at an angle to the direction of traverse of an Eberbach shaker reciprocating at 30 cycles per min. The angle was held at approximately 80° to the horizontal.

D. Measurements of Enzyme Activity

The yeast beta-galactosidase in cell-free preparations was inactivated by the deionized water provided. Therefore, double distilled water was prepared in a pyrex still containing deionized water, H_2SO_4, and $KMnO_4$ in the first flask, and NaOH in the second flask. The

distillate from the second flask was employed for the preparation of all solutions excepting media.

1. Ortho-nitrophenol-beta-D-galactoside (ONPG) method for measurement of beta-galactosidase

The chromogenic substrate, ONPG, is hydrolyzed by emulsin lactase (25), by the E. coli lactase (27) and by the yeast lactase (9). Employing this substrate, S. fragilis beta-galactosidase was assayed by the following procedure: 4 ml of assay mix, 1 x 10^{-3} M in ONPG, and containing a buffer to be specified, was dispensed into a calibrated colorimeter tube. Following temperature equilibration at 30°C, an aliquot of enzyme solution was added, and the tubes incubated at 30°C. At regular intervals, readings were taken on a Klett-Summerson Colorimeter using a blue filter and an air blank. The enzyme activity is expressed as the number of Klett units change caused by 1 ml of enzyme preparation during a period of one minute. Multiplying this number by 2.4 converts these arbitrary units into $\frac{m\mu M \text{ ONPG hydrolyzed}}{ml \times min}$ (28).

2. Manometric methods for measurement of beta-galactosidase

The need for testing enzyme activity on beta-galactosides other than the chromogenic substrate required the use of other assay procedures. Manometric assay systems for the products of beta-galactosidase activity are described in (a) and (b) below. In the absence of added beta-galactosidase, neither assay system exhibits activity on any of the beta-galactosides employed.

A conventional Warburg apparatus held at 30°C was employed. The vessels were shaken through a 7 cm arc at 110 cycles per minute. Enzymatic activity is expressed as μ M substrate hydrolyzed per minute by one ml of enzyme solution.

a. Measurement of glucose released by beta-galactosidase hydrolysis. This measurement (3) is based on the fact that in the presence of azide (28), Torula monosa releases 2 moles of CO_2 per mole of glucose fermented. 18 hour aerated cultures of Torula monosa were grown in the complete medium supplemented with 2 % dextrose. The cells were harvested aseptically, resuspended in one-half the original volume of nutrient medium, and then flushed with nitrogen for 3 hours to decrease their endogenous metabolism. This suspension can be stored under refrigeration for one week.

For use in assay, the cells were harvested, washed twice with cold double distilled water, and suspended in three times their volume of 0.1 M potassium phosphate buffer, pH 6.8. An aliquot of beta-galactosidase was added to the side arm of each Warburg vessel. Additions of 0.5 ml Torula monosa suspension, lactose to a final concentration of 0.1 M and 0.05 M potassium phosphate buffer of pH 6.8, were made to the main compartment. The final volume in each cup was 2 ml. After the vessels were flushed with nitrogen and shaken at 30°C for thermal equilibration, the enzyme was tipped into the center compartment, and measurements taken of the rate of gas released.

b. Measurement of galactose released by beta-galactosidase hydrolysis of galactosides. A similar type of assay for galactose released from galactosides other than lactose has been devised, employing galactose adapted cultures of S. cerevisieae, strain K.

The induction of galactozymase in S. cereviseae, strain K, is difficult to control in the complete medium. It can, however, be routinely accomplished by growing the cells in Davies medium supplemented with 0.25 % enzyme hydrolyzed casein, 1 % ethanol, and 2 % galactose purified

by the biological method. Inoculation and growth conditions have been described in the previous section. The cells were harvested when an O.D. of 1.0 had been attained, washed twice with cold double distilled water, and suspended in twice their volume of 0.1 M potassium phosphate buffer, pH 6.8.

The O.D. at which the cells were harvested was found to be critical for high galactozymase content. Cells from cultures below O.D. 0.8 and above O.D. 1.3 possessed little galactozymase.

Washed strain K cells placed under anaerobic conditions showed little or no endogenous metabolism and fermented galactose at an increasing rate. Addition of 1×10^{-3} M sodium azide caused the fermentation rate to remain constant (29), and under these conditions, 2 moles of CO_2 were released per mole of galactose.

c. Conditions for linear relationship between enzyme concentration and rate of CO_2 evolution. With these two indirect assay methods, it is necessary that galactose or glucose production be the rate limiting step in CO_2 evolution if the rate of CO_2 production is to be proportional to enzyme concentration. To achieve these conditions, the activities of S. cerevisieae, strain K, on galactose and Torula monosa on glucose, were measured before each experiment. Concurrently, beta-galactosidase activity on lactose and on phenyl-beta-D-galactoside was measured employing varying enzyme concentrations. An enzyme concentration was then chosen such that the rate of CO_2 production would be one-tenth that which the assay system cells could produce under conditions of substrate saturation.

3. Alpha-phenyl glucoside method for measurement of maltase activity

Alpha-glucosidase activity was measured by a colorimetric method.

The test is based upon the deacidation coupling of quinonechloroimides and phenol, a reaction which was first noted by Hirsch (30), and discussed in detail by Gibbs (31). The colored compound indophenol is produced according to the equation:

$$O{:}C_6H_4{:}NCl + C_6H_5OH \longrightarrow O{:}C_6H_4{:}N.C_6H_4OH + HCl.$$

Because of the sensitivity of the reaction and the stability of the products, 2,6 dichloro- and 2,6, dibromoquinonechloroimides were found to be useful for the quantitative determination of phenol (31). Sanders and Sager (32,33) and Horwitz (34) incorporated the indophenol technique in an assay for phosphatase activity. Spiegelman (28) has modified their method for the measurement of alpha-glucosidase, and the test as finally evolved, is a modification of the Spiegelman method.

a. Reagents employed.

1. pH 6.8, 0.1 M potassium phosphate buffer.
2. 0.8 % alpha-phenyl glucoside, aqueous solution.
3. cysteine, 40 mgm in 10 ml double distilled water.
4. pH 9.8 borate buffer, prepared by adding to 100 ml 0.2 M KCl, 100 ml 0.2 M boric acid, 82 ml 0.2 M NaOH, and double distilled water to make a final volume of 400 ml.
5. 2,6, dibromoquinonechloroimide (BQC), 40 mgm dissolved in 10 ml absolute ethanol is stored in a dark bottle under refrigeration. The compound is unstable and solutions were prepared daily.
6. phenol standard, 100 ml of stock standard is prepared by adding water to 1.0 gm phenol in a volumetric flask. A 1:10 dilution of the stock standard constitutes the working standard.

b. Procedure. 0.4 ml of reagent mix consisting of 0.2 ml potassium phosphate buffer, 0.1 ml of a 1:10 dilution of cysteine solution, and 0.1 ml alpha-phenyl glucoside are dispensed into a standardized Klett tube, and incubated at 30°C for temperature equilibration. 0.05 ml to 0.2 ml enzyme solution is then added. Following incubation at 30°C for a suitable period of time, 4 ml borate buffer are added to stop the enzyme reaction and to adjust the tube contents to the pH optimum for the indophenol reaction. After an addition of 0.2 ml BQC solution, the tube is thoroughly shaken, and the color allowed to develop at room temperature for 30 to 90 minutes. Colorimetric measurements are made with a Klett-Summerson Colorimeter equipped with a red (620) filter, and zeroed with an air blank. BQC under these conditions gives a color reaction. Therefore, a blank consisting of 0.3 ml potassium phosphate buffer, 0.1 ml 1:10 dilution of cysteine, 4 ml borate buffer, and 0.2 ml BQC solution is prepared simultaneously with the reaction tubes. The phenol content is determined from these two readings with the aid of a standard curve.

A range of 0 to 100 micrograms of phenol per tube is employed to construct this standard curve (Figure 2). The following additions are made to each calibrated Klett tube: 0.1 ml phenol solution, 0.2 ml potassium phosphate buffer, 0.1 ml of a 1:10 dilution of cysteine, 4 ml borate buffer, and 0.2 ml BQC solution. The tubes are shaken, incubated at room temperature for 30 to 90 minutes, and read in the colorimeter as previously described. Standard curves constructed as described are linear to 30 micrograms of phenol. Higher quantities of phenol yield Klett readings which fall below the expected values. The system is not limited by BQC since higher concentrations of BQC do not obviate the difficulty. Further, as had already been noted by Sanders and Sager (32), high concentrations of BQC interfere with the reaction. However, tubes containing higher phenol

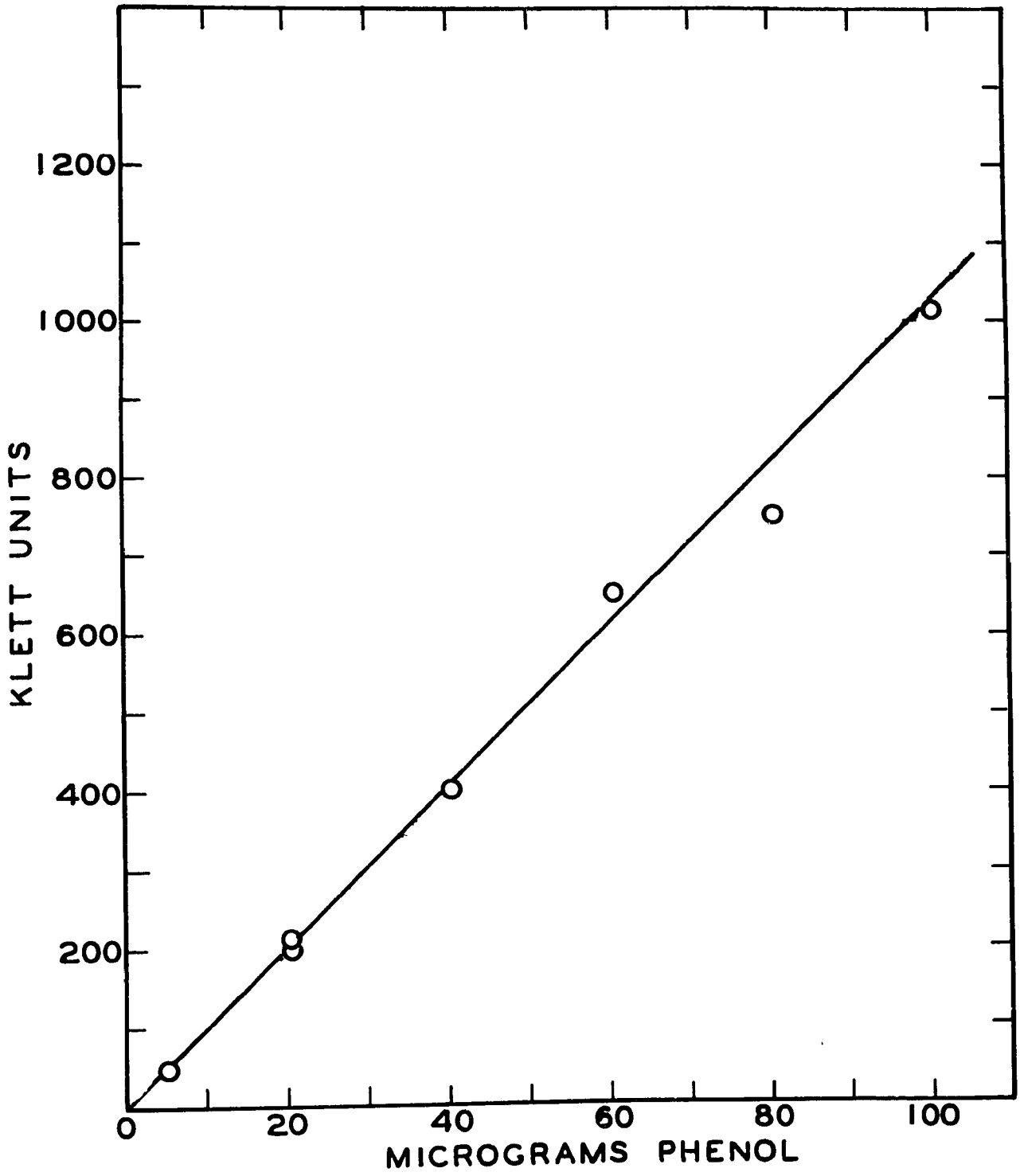


Fig. 2. Relation between phenol concentration and klett units in alpha-glucosidase test.

concentrations give satisfactory readings when diluted with the blank solution as follows:

Klett reading undiluted tube	Dilution factor
240-270	1:2
270-350	1:5
350 and higher	1:10

Higher dilutions yield Klett values greater than expected from linearity.

When this method is employed, the rate of indophenol production is proportional to the amount of enzyme in the tube, and enzyme activity is found to be constant over a one hour interval.

However, the method as herein described is subject to interference of color development by two components of the system. The first component is cysteine which is oxidized by BQC. The latter compound may thereby be reduced below the saturation level for the indophenol reaction. But cysteine has since been found unnecessary for enzyme stabilization and may be dispensed with. Colored compounds, formed when protein is present in high concentration, can also interfere with the assay. In this event, the Sanger and Sager (32) technique of deproteinization before BQC addition, and n-butyl alcohol extraction of the indophenol may be employed.

E. Preparation of Cell-free Extracts for Study of Beta-galactosidase

S. fragilis, strain Y 665, was inoculated as described into the complete medium supplemented with 1 % ethanol. When cells induced for beta-galactosidase were desired, galactose, purified by the biological method, was added to a final concentration of 2 %. Cultures were incubated with

aeration at 30°C for 10 to 12 hours. The cells were harvested, washed twice with 0.05 M potassium phosphate buffer of pH 7.0, and resuspended in the same buffer to a density of 100 mgm wet weight per ml. Glutathione, 0.5 mgm per ml of suspension, was added for enzyme stabilization, and the cells were fragmented in a Raytheon 10 Kc sonicator for 20 minutes at 1.2 amps and 65 to 72 volts. Whole cells and debris were removed by centrifugation for 20 minutes in an SS1 Sorvall at 7000 rpm. The supernatant was stored in a deep freeze until tested.

In addition to the above extracts, a different type of preparation from induced S. fragilis has also been employed. Beta-galactosidase which had been partially purified by acetone precipitation followed by ammonium sulfate fractionation was kindly supplied by Dr. G. Loggers of the Koninklijke Nederlandscher Gist-En Spritisfabriek Laboratory in Delft. In this study, it is designated as the Delft preparation. For experimental work, it was dissolved in double distilled water or 0.1 M pH 6.8 potassium phosphate buffer by means of grinding in a cold mortar with a chilled pestle. The insoluble non-active precipitate which remained after grinding was removed by centrifugation in an SS1 Sorvall for 20 minutes at 7000 rpm. The supernatant possessed an activity of 200 expressed as mμM ONPG hydrolyzed/ (ml x min).

III. EXPERIMENTAL RESULTS

A. Properties of S. fragilis Beta-galactosidase

This thesis is concerned with a comparison of induced and constitutive enzyme synthesis. The beta-galactosidase of S. fragilis was chosen, not only to compare such results where possible with those of the beta-galactosidase in E. coli, but also to compare the specificities of their induction systems. In order to undertake such a study, the following information must be available:

1. requirements for full enzymatic activity to ensure accurate and reliable assays,
2. whether or not the enzymes produced constitutively and under induction are identical, and
3. whether one or more protein species possessing the same enzymic function are synthesized.

Because such information was either unknown, or incompletely delineated, it was necessary to study the properties of the enzyme. Experiments have been performed with beta-galactosidase from induced and constitutively grown cells in order to answer the second question raised above.

1. Effect of pH on the stability of the enzyme

Solutions of the Delft preparation in 0.1 M potassium phosphate buffers of pH 4.5, 5.5, 6.5, 7.0, 7.5 and 8.0 were prepared as described above. Assays for enzyme activity were made immediately and after 24 and 48 hours of storage at 0°C. As seen in Table 1, the enzyme is stable at pH's of 7.0, 7.5, or 8.0.

TABLE 1

STABILITY OF YEAST BETA-GALACTOSIDASE TO pH

pH	mμM ONPG hydrolyzed / (ml x min)		
	0 hr	after 24 hr	after 48 hr
4.5	125	12.8	6.0
5.5	346	152.5	78.7
6.5	538	440	264
7.0	538	600	607
7.5	655	672	672
8.0	571	660	522

Beta-galactosidase was suspended in potassium phosphate buffers at varying pH's, and stored at 0°C. Assays for ONPG hydrolytic activity were made in 0.1 M potassium phosphate buffer, at pH 7.5, and 10^{-3}M in $MgSO_4$, at 0 time, and after 24 and 48 hours of storage.

2. Ions as activators of enzyme activity

It had been reported that magnesium and potassium ions are activators for this enzyme (8,9). For reliable determinations of enzyme activity, it was pertinent to enquire into the possible additive effect of these ions, the optimal concentrations for activation, and the absolute requirements for one or both.

Contaminating ions were removed by dialysis against 0.6 % Versene (sodium ethylene diamine tetraacetate), adjusted to a pH of 7.4 with 1.0 M KOH (35). One volume of enzyme preparation was dialyzed with stirring for 12 hours at 5°C against 500 volumes of Versene solution. The Versene dialysis was followed by five dialyses of one to two hours against 500 volumes triple distilled water. Assays of enzyme activity were made by the ONPG method.

As seen in Table 2, all enzyme preparations, from either induced or non-induced cells, demonstrated an absolute requirement for magnesium ion following dialysis, whereas non-dialyzed extracts were activated to varying degrees by the ion. The effect of magnesium, manganese, and calcium ion concentration on enzyme activity is shown in Figure 3. Manganese is not as effective as magnesium at low concentration; calcium ion has little effect at any concentration.

When studying the effect of potassium and sodium ions on yeast beta-galactosidase, assays were performed in ONPG assay mix buffered with 0.01 M aqueous triethanol amine (Tris) solution adjusted to pH 7.5 with HCl and containing 1×10^{-3} M $MgSO_4$. Varying concentrations of KCl and NaCl and 1×10^{-3} M methionine were added to the assay mix. As shown in Table 2, all dialyzed yeast beta-galactosidase preparations demonstrate a requirement not only for magnesium, but also for sodium or potassium ion.

TABLE 2

MAGNESIUM, AND POTASSIUM OR SODIUM ION REQUIREMENT

FOR BETA-GALACTOSIDASE ACTIVITY

Cation additions to ONPG assay mix *			mμM ONPG hydrolyzed / (ml x min) Extract constitutive cells		Extract induced cells	
10^{-3}M Mg	10^{-2}M K	10^{-2}M Na	dialyzed	untreated	dialyzed	untreated
-	-	-	0	0	0	6.7
+	-	-	4.8	0	8.9	16.8
-	+	-	4.6	389	61.5	2280
+	+	-	451	312	2426	1920
+	-	+	432	672	2480	1850

* Assay mix before additions consisted of 10^{-3} M ONPG dissolved in 0.01 M Tris buffer, adjusted to pH 7.5 with HCl. Extracts prepared from induced and non-induced cells were Versene-triple distilled water dialyzed as described, and assayed for enzyme activity on ONPG.

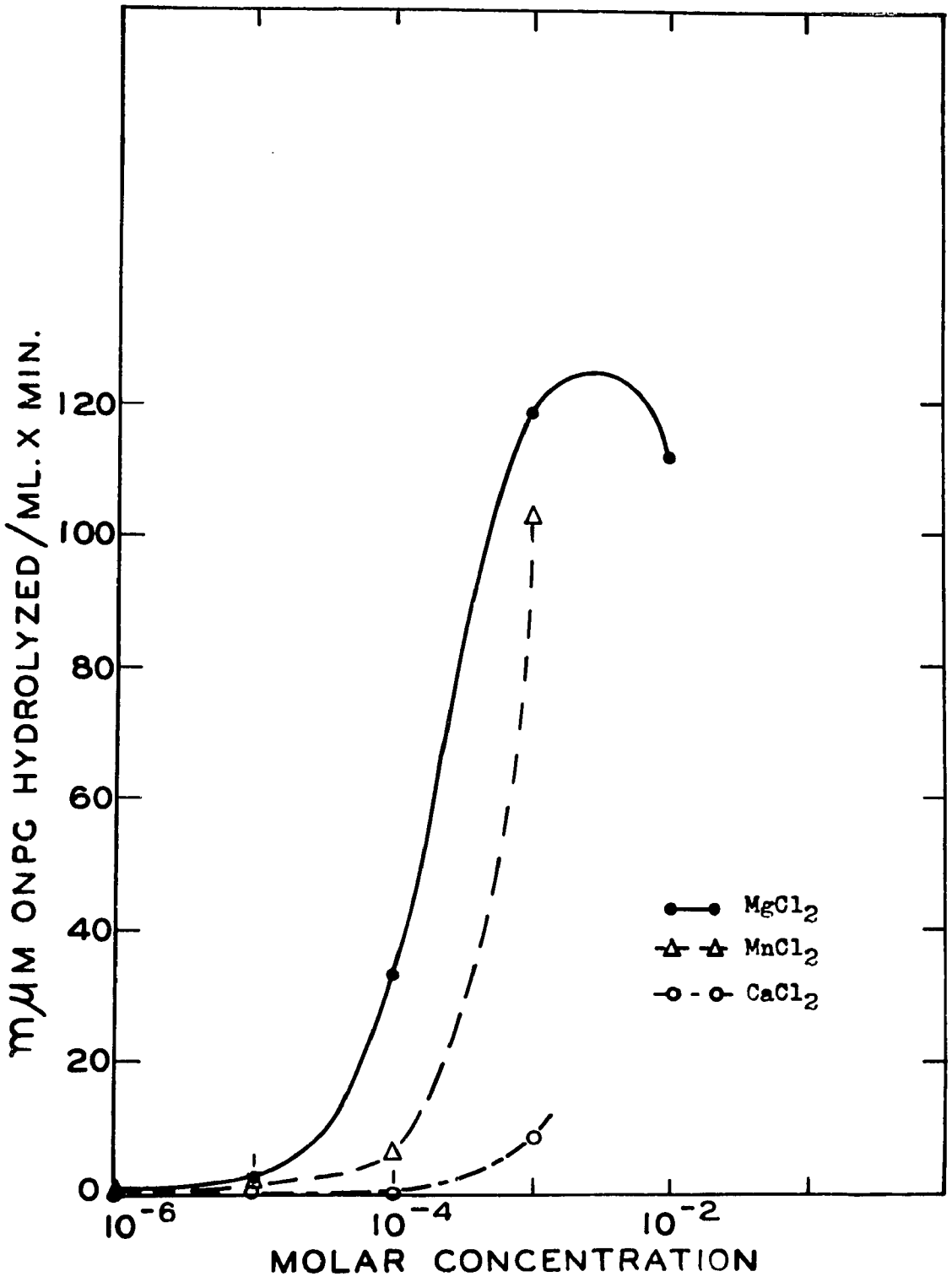


Fig. 3. Effect of divalent ions on activity of beta-galactosidase in extract of induced cells. Assays performed in 0.05M Na_2HPO_4-KH_2PO_4 buffer of pH 7.5 and 0.001M in ONPG.

Figure 4 demonstrates the relationship between enzyme activity and concentration of these two monovalent cations. Although higher enzyme activities are realized in the presence of sodium ion, the potassium ion produces its maximum activation at a lower concentration than does the sodium ion.

Sodium ion is a better activator than potassium, but it also has an inactivation effect, as shown in Tables 3 and 4. Preliminary experiments performed with varying concentrations of the enzyme in Tris buffer confirm this finding. Whereas the enzyme activity with KCl is proportional to enzyme concentration, the activity in NaCl is not proportional to enzyme concentration with low levels of the enzyme. It follows that with low enzyme concentrations, it would be virtually impossible to measure a sodium ion activation. The data in Figure 4 were obtained by the use of high enzyme concentrations and assaying in the presence of 1×10^{-3} M methionine (see below).

The magnesium requirement suggested the possible involvement of a phosphorylation reaction. However, as demonstrated in Table 5, the addition of inorganic arsenate did not increase the rate of hydrolysis of ONPG. Similar results have been obtained from experiments with inorganic phosphate.

On the basis of the ion requirements for maximum enzyme activity, the maximum rate of ONPG hydrolysis should be realized in ONPG assay mix buffered with 0.1 M sodium phosphate buffer, and supplemented with 1×10^{-3} M $MgSO_4$. However, under these conditions, enzyme activity rapidly decreased during the assay. On testing beta-galactosidase action in a variety of buffers, the greatest stability was found in 0.1 M Na_2HPO_4-KH_2PO_4 buffer, of pH 7.5 (see Table 3). However, this was not a reproducible condition for stability. Addition of the products of the reaction at 1×10^{-4} M,

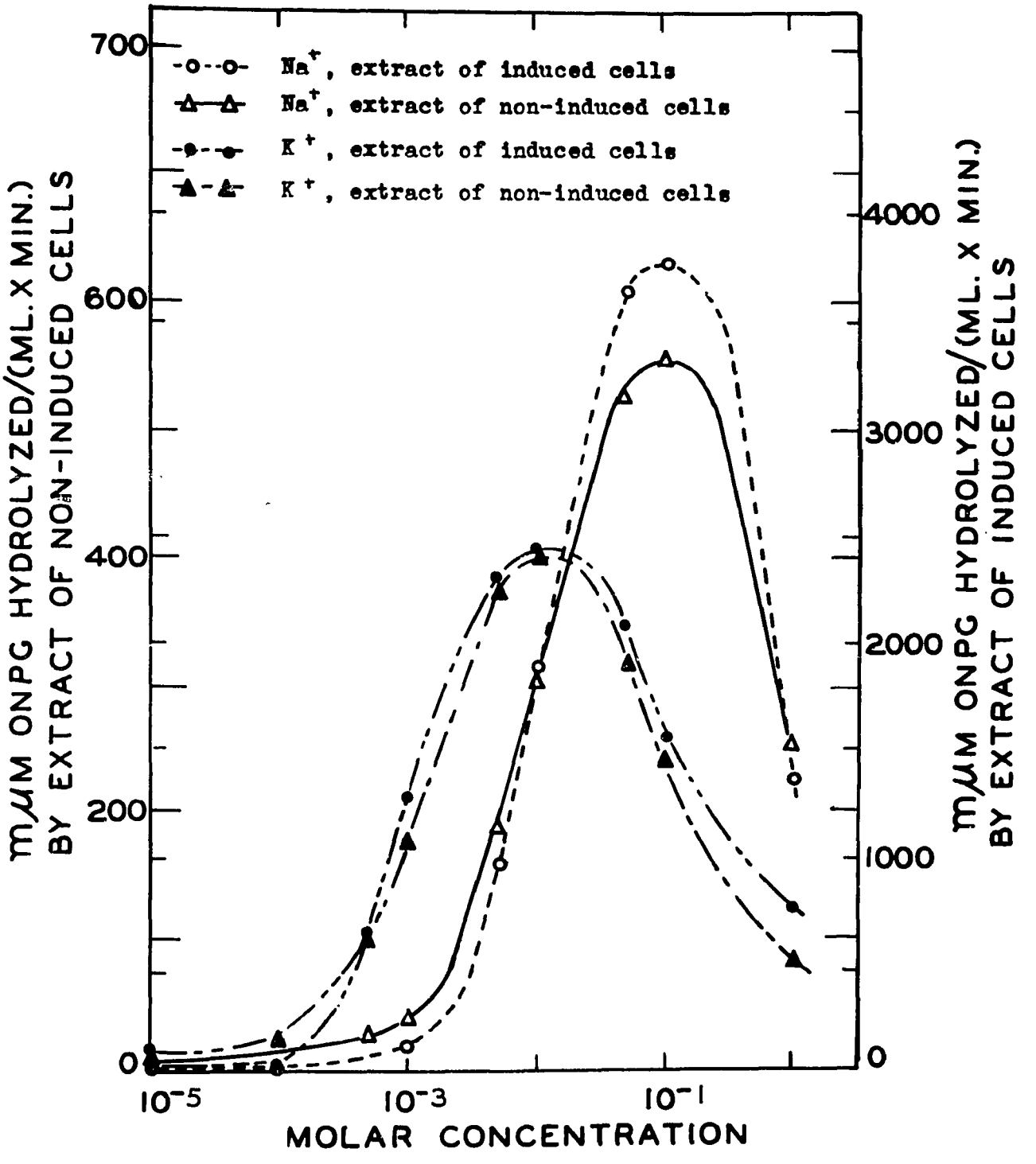


Fig. 4. Effect of potassium and sodium ion concentration on beta-galactosidase activity.

TABLE 3

EFFECT OF BUFFER IN ONPG ASSAY MIX ON BETA-GALACTOSIDASE STABILITY DURING ASSAY

Enzyme	mμM ONPG hydrolyzed / (ml x min) at designated time in mix with 0.1 M buffer of pH 7.5											
	Na_2HPO_4-NaH_2PO_4				Na_2HPO_4-KH_2PO_4				KH_2PO_4-K_2HPO_4			
Constitutive, versene-	1'	2'	3'	4'	2'	3'	4'		3'	7'	8'	10'
water dialyzed	1200	1020	480	360	3600	3480	2880		535	530	384	336
Constitutive,	2'	4'	6'	8'	2'	3'	4'		2'	4'	6'	
untreated	3120	2690	2400	1740	5650	5290	5530		705	495	120	
Induced, versene-water	8'	16'	24'	32'	10'	20'	30'		10'	20'	30'	
dialyzed	54	50.5	48	39	48	48	48		21.6	21.6	21.6	
Induced	4'	8'	12'	16'	10'	13'	20'	30'	10'	20'	30'	40'
untreated	174	153	99	81	106	120	108	101	91.4	86.5	65	65

Assays were performed at 30°C. Assay mixes were prepared by dissolving ONPG and $MgSO_4$ to final concentrations of 1×10^{-3} M in the above buffers.

TABLE 4

METHIONINE STABILIZATION OF BETA-GALACTOSIDASE ACTIVITY ON ONPG

pH 7.5 buffer in assay mix [a]	Methionine 1×10^{-3} M (final conc.)	$m\mu$ M ONPG hydrolyzed / ml x min (initial activity)	% loss of activity after 15 min 30°C [b]
0.1 M K_2HPO_4-KH_2PO_4	-	178	8
	+	245	10
0.014 M K_2HPO_4-KH_2PO_4	-	202	43
	+	327	10
0.1 M Na_2HPO_4-KH_2PO_4	-	186	70
	+	336	10
0.1 M Na_2HPO_4-NaH_2PO_4	-	33.6	100
	+	96	50

The enzyme employed in these experiments was a 0.1 M potassium phosphate solution of the Delft preparation.

[a] Assay mix consisted of designated buffer 1×10^{-3} M in $MgSO_4$ and 1×10^{-3} M in ONPG.

[b] With the Delft preparation, losses in enzyme activity greater than 15 % are considered significant.

TABLE 5

EFFECT OF INORGANIC ARSENATE ON ONPG HYDROLYSIS

Additions to ONPG Assay Mix *		mμM ONPG hydrolyzed / ml x min
Na_3AsO_4	1×10^{-2} M	144
NaCl	3×10^{-2} M	139
K_3AsO_4	1×10^{-2} M	151
KCl	3×10^{-2} M	192
none		0·31

* Assay mix consists of 0.01M Tris buffer of pH 7.5 and 1×10^{-3} M in both $MgSO_4$ and ONPG.

The enzyme preparation employed was purified by starch column ionophoresis (see III, 4), followed by elution in triple distilled water.

had no effect on the rate of ONPG hydrolysis; nor was the level of ONPG limiting. Indeed, the losses in activity were directly attributable to enzyme inactivation by incubation at 30°C. Enzyme solutions incubated at 30°C for periods comparable to those employed in the assay, resulted in similar losses of activity.

In view of this result, a variety of possible stabilizers, including glucose, galactose, methyl-beta-D-thiogalactoside, azide, glutathione, cysteine, methionine and alanine were tested for their effect on enzyme activity. Galactose and glucose below 0.01 M final concentration had no effect on the system. Methyl-beta-D-thiogalactoside enhanced the instability, and contrary to Connor's findings (9), azide also increased the rate of enzyme inactivation. However, cysteine at 2×10^{-2} M, methionine at 1×10^{-3} M, alanine at 5×10^{-3} M, and glutathione at 2×10^{-2} M each markedly decreased the rate of inactivation when ONPG assays were performed in an assay mix buffered with 0.1 M potassium phosphate buffer at a pH of 7.5. Methionine at 1×10^{-3} M also effected a reactivation of enzyme inactivated by incubation at 30°C, or storage at 0°C.

Table 5 shows the stability of ONPG hydrolytic activity in a variety of buffers with and without 1×10^{-3} M methionine. The reproducibility of high activity and stability in 0.014 M potassium phosphate buffer supplemented with magnesium and methionine both at 1×10^{-3} M was greater than that in a similar system buffered with Na_2HPO_4-KH_2PO_4. Therefore, for routine assays of enzyme activity by the ONPG method, the assay mix consisted of 0.014 M potassium phosphate buffer at a pH of 7.5 supplemented with methionine to 1×10^{-3} M, $MgSO_4$ to 1×10^{-3} M, and ONPG to 1×10^{-3} M.

Under these conditions, enzyme concentration is directly proportional to mμM ONPG hydrolyzed per min, and mμM ONPG hydrolyzed per

ml of enzyme solution are directly proportional to time. These relationships exist only when Klett readings below 250 are employed.

3. pH optimum for S. fragilis beta-galactosidase activity

The effect of pH on enzyme activity was studied by measuring the extent of hydrolysis of ONPG. 0.01 M potassium phosphate buffers at pH's of 5.5, 6.0, 6.3, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8 were each supplemented with final concentrations of 1×10^{-3} M $MgSO_4$, 1×10^{-3} M methionine, and 1×10^{-3} M ONPG. The methodology previously described for ONPG assay was not applicable for this experiment because there is a pH effect on the color of ortho-nitrophenyl. Therefore, following incubation of assay mix and enzyme at 30°C for a specified time interval, 5 ml of 0.2 M NaOH were added to a reaction tube. The tube was then shaken and read in the colorimeter within 0.3 min of the sodium hydroxide addition. Short incubation times reduced the possibility of enzyme inactivation. As seen in Figure 5, beta-galactosidases from induced and non-induced cells both have a fairly sharp pH optimum at 6.8. The low activities realized below pH 6.5 may in part be caused by enzyme inactivation (see Table 1).

4. Starch column ionophoresis

Starch column ionophoresis is useful not only for purification purposes, but also as a tool in determining whether a specific type of enzyme activity is comprised of more than one protein species, or whether enzymes from two sources are identical. For these reasons, crude extracts prepared from induced and non-induced cells have been subjected to this treatment.

The technique as described by Rotman and Spiegelman (6) has been slightly modified. When input material was mixed with dry starch, the quantity of starch employed was such that a very dry, crumbling paste was

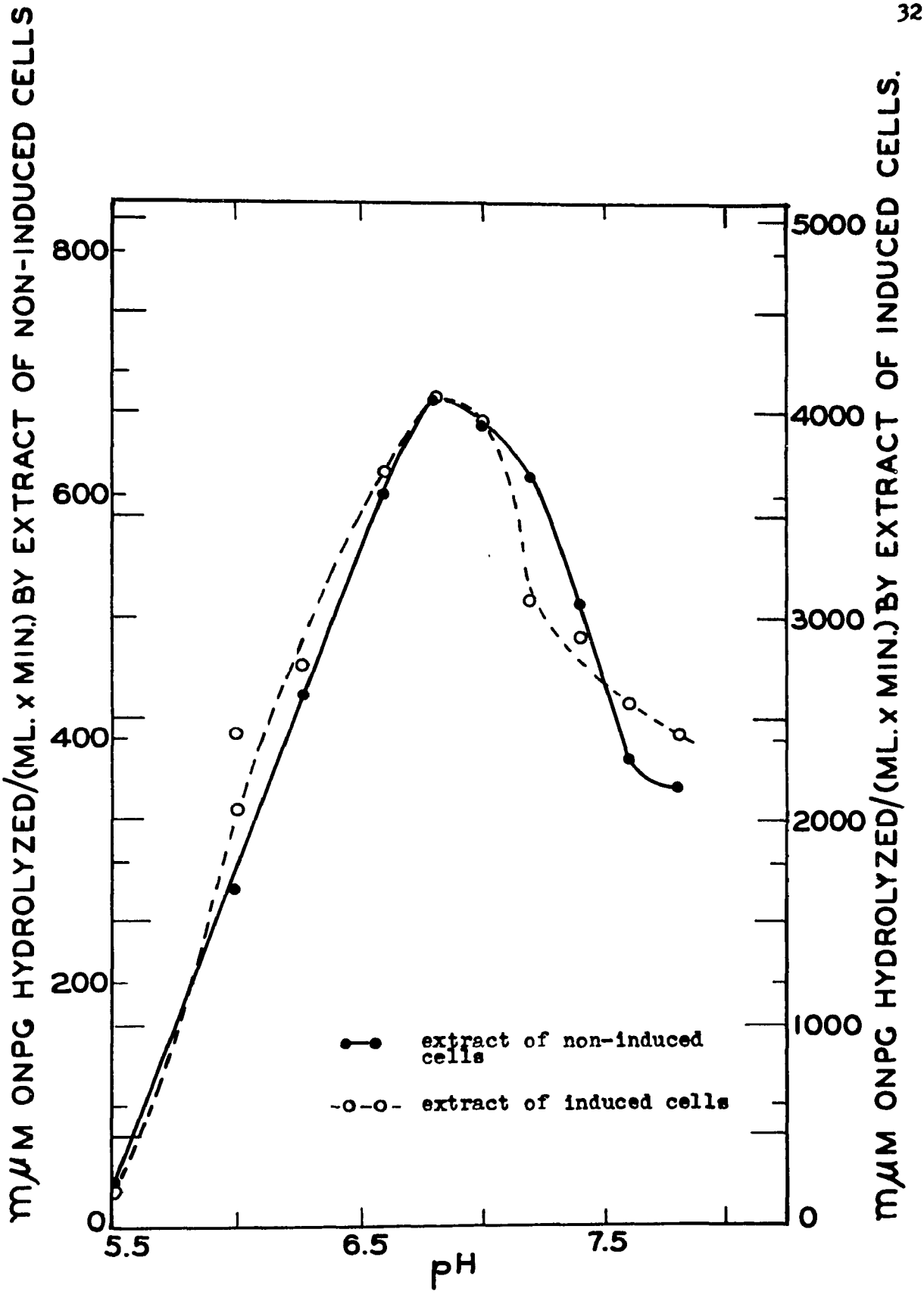


Fig. 5. Effect of pH on beta-galactosidase activity

formed. This procedure minimized trailing; the enzyme was found to extend over a one to two centimeter zone in the columns employed. In addition, a length of blotting paper was inserted between the column and its lucite cover to keep droplets of condensed water from falling back onto the starch.

0.072 M potassium phosphate buffer of pH 7.0 to 7.2, supplemented with 1 x 10^{-3} M methionine, was routinely used. The potential difference across the column was 300-250 volts, and the current averaged 7-9 milliamps for a column 2 cm wide and 0.85 cm deep. Because it is desirable to obtain sharp peaks of enzyme activity, columns wider than 2 cm have not been employed. Following development of the column for 10 to 20 hours, the position of the enzyme was located by placing a small volume of starch from each centimeter along the length of the column into 0.1 ml of the ONPG assay mix on a spot plate. 1.0 cm wide strips were cut out in the region of the enzyme, and 2 cm strips elsewhere. Each strip was placed in a test tube, 2 ml of 0.05 M potassium phosphate buffer of pH 7.5, 1 x 10^{-3} M in methionine, were added, and the tubes were shaken at 4°C for 40 minutes. The starch was then allowed to settle for 15 minutes, and the supernatant decanted.

The supernatant was analyzed for beta-galactosidase activity and protein content. The latter analysis was accomplished by Lowry's (36) method, the former by both the ONPG method and the manometric methods previously described.

In starch columns buffered with 0.072 M potassium phosphate buffer of pH 7.0 to 7.2, beta-galactosidase from crude extracts of induced cells moved towards the positive pole at an average speed of 0.66 to 0.76 cm per hour. The rate of movement was decreased to 0.2 cm per hour when a sodium pyrophosphate buffer of pH 8.4, 0.025 M in phosphate and 1 x 10^{-3} M in methionine was employed. Recovery of enzyme activity ranged from 80% to

100 %, as measured by the ONPG method.

Eluates from columns of induced cell extracts were tested for hydrolysis of ONPG and lactose in an effort to determine whether or not the hydrolytic activity for both substrates resides in the same enzyme molecule. The results of a typical experiment are given in Figure 6. Each eluate possessing hydrolytic activity on ONPG could also hydrolyze lactose. It is therefore probable that one type of enzyme molecule can hydrolyze both substrates.

Figure 6 also demonstrates one peak of hydrolytic activity on both substrates, indicating the presence of only one type of protein possessing activity on beta-galactosides in induced cells. However, the possibility that there are other highly unstable beta-galactosidases is not completely eliminated.

The mobilities of the beta-galactosidase in extracts prepared from induced and non-induced cells have been compared. For this purpose, three columns were simultaneously developed in 0.072 M potassium phosphate buffer. These columns contained, respectively, 1 ml extract from induced cells, 1 ml extract from non-induced cells, and a mixture of 0.5 ml of each of the two types of extracts. As seen in Figure 7, the movement of beta-galactosidase was similar in the three columns. Thus, the constitutive and inducible beta-galactosidases cannot be distinguished by this test.

5. Hydrolysis of galactosides by intact cells and cell-free extracts

An examination of the specificity of enzyme activity of beta-galactosides was desirable for the following reasons:

(i) to test the identity of constitutive and induced enzyme

(ii) to compare the specificity of beta-galactosidase activity with the specificity of the induction process,

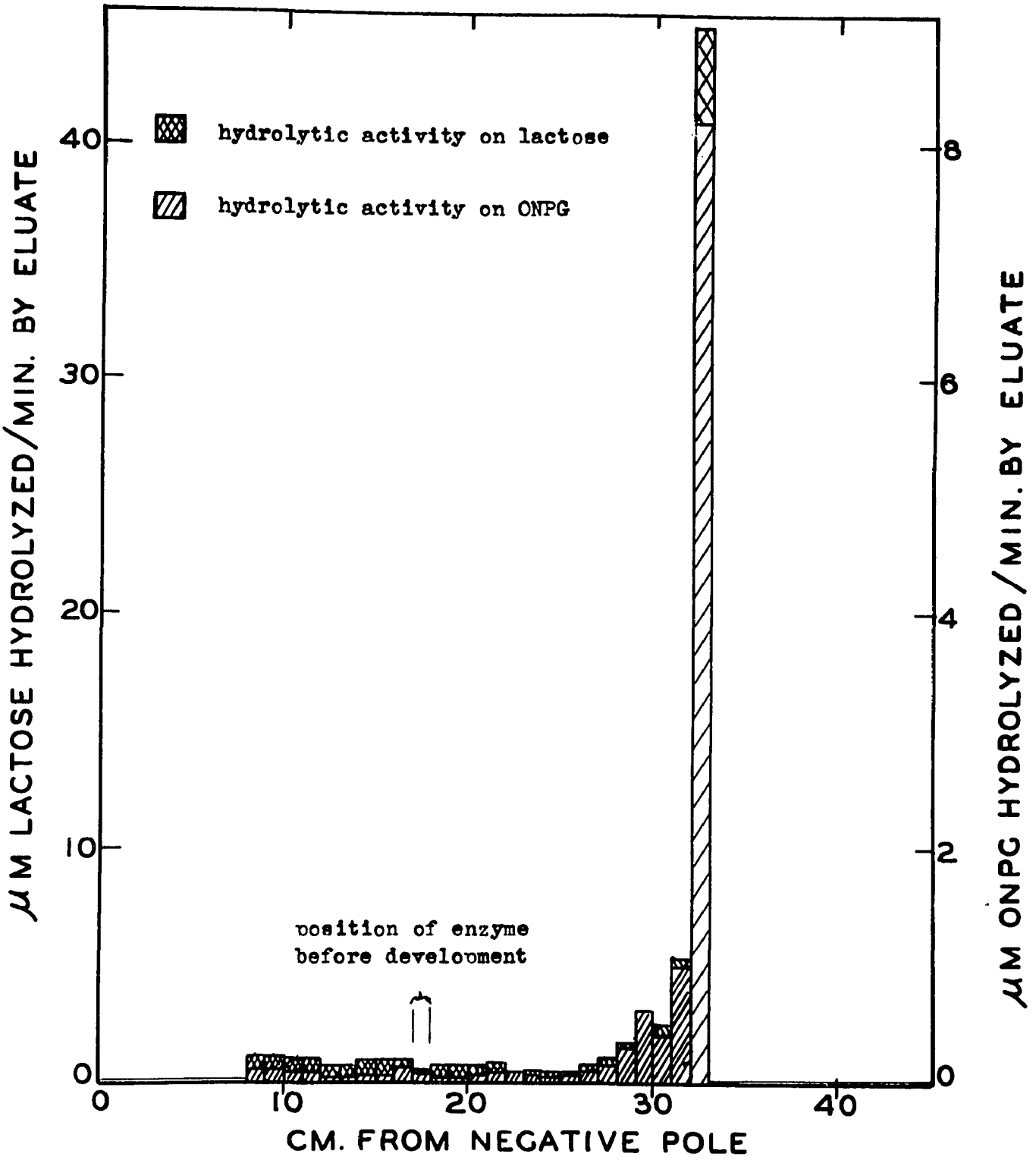


Fig. 6. Location of lactose and ONPG hydrolytic activities following starch column ionophoresis. Column was developed for 21.5 hr. Lactose hydrolysis measured manometrically in 0.05 M potassium phosphate buffer of pH 6.8. Activity on ONPG measured colorimetrically in 0.014 M potassium phosphate buffer, pH 7.5.

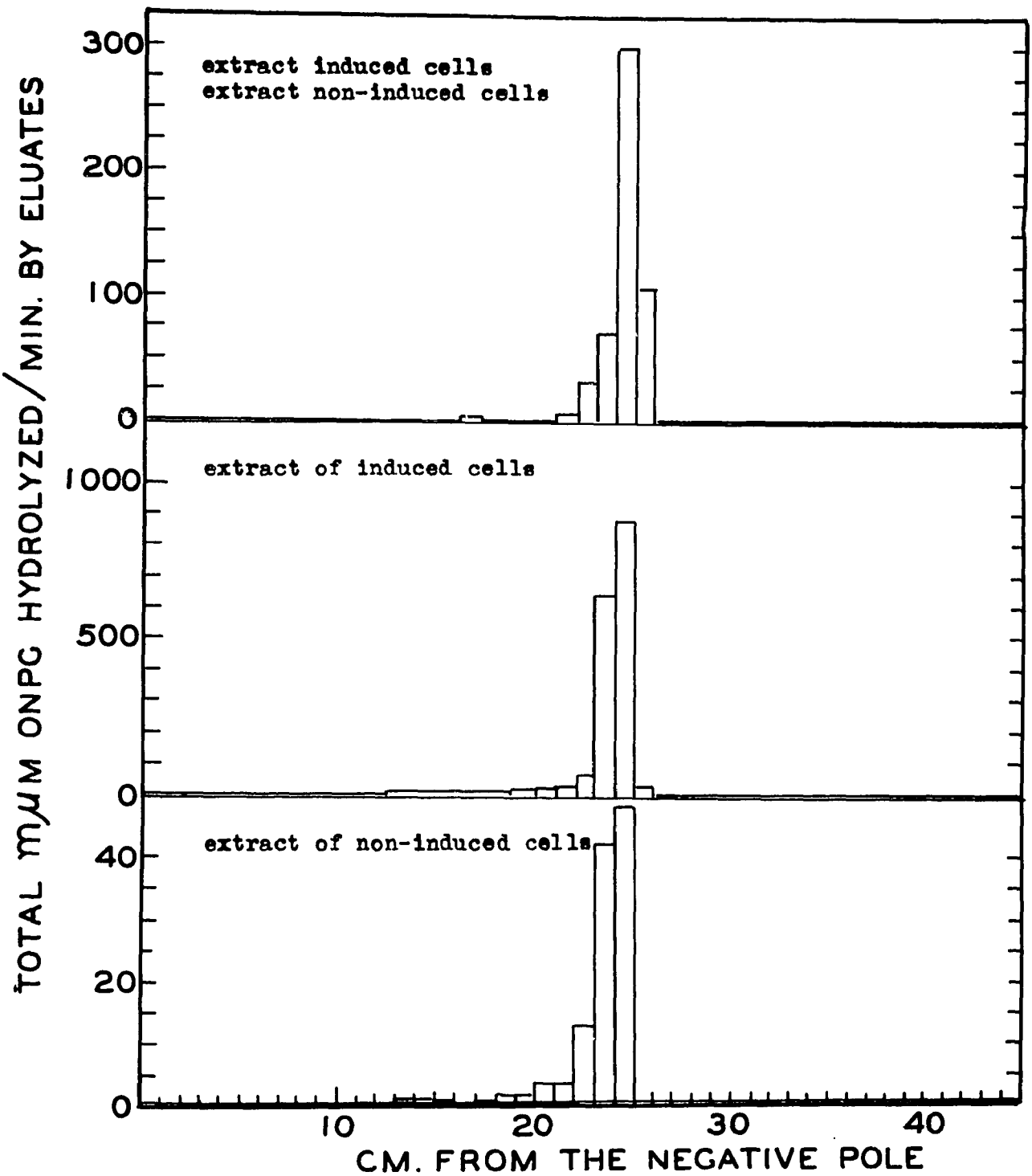
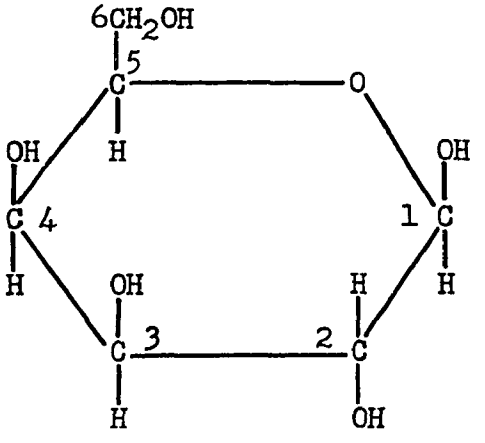


Fig. 7. Starch column ionophoresis of beta-galactosidases in extracts of induced and non-induced cells. Columns were developed for 14 hours. Before development, enzyme samples were between 20 cm and 21 cm.

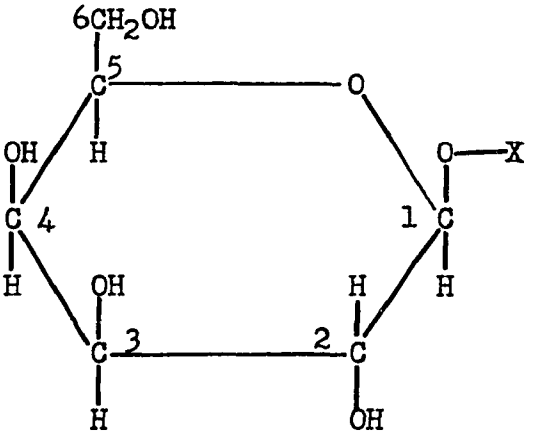
(iii) to compare the specificities of beta-galactosidases from S. fragilis and E. coli,

(iv) to test the effect of induction on crypticity of beta-galactosidase in S. fragilis.

The Haworth configurations of the galactose molecule and of a beta-galactoside are given below.



beta-D-galactose



X-beta-D-galactoside

Specificity of beta-galactosidase activity was tested with whole cells and cell-free extracts employing the compounds listed in Table 6. The rates of hydrolysis were measured by the manometric procedures described in Methods.

For extracts and whole cells of S. fragilis, strain Y 665 was cultured under aerobic conditions at 30°C as previously described. When the culture had an O.D. of 1.0, the cells were harvested, washed twice with cold double distilled water, and weighed. A suspension of 100 mgm wet weight per ml was prepared in 0.05 M potassium phosphate buffer of pH 6.8. An aliquot of the suspension was converted into a crude extract by the sonication procedure previously described. The remainder was centrifuged for 20 minutes

TABLE 6

DESCRIPTION OF GLYCOSIDES

Glycoside	Chemical substitution
Galactose	Hydrogen atom on C_1 in position X
Lactose	glucose C_4 molecule on C_1 in position X
Phenyl-beta-D-galactoside	phenyl group on C_1 in position X
Methyl-beta-D-galactoside	methyl group on C_1 in position X
n-butyl-beta-D-galactoside	n-butyl group on C_1 in position X
Phenyl-alpha-D-galactoside	phenyl group on C_1 in position X in the alpha configuration
Ortho-nitrophenyl-beta-D-galactoside	ortho-nitro phenyl group on C_1 in position X
Methyl-alpha-D-galactoside	methyl group on C_1 in position X in the alpha configuration
Melibiose	linkage from C_6 of glucose molecule in position X to C_1 through the alpha configuration
Methyl-beta-D-thiogalactoside	O-X replaced by S-methyl group in the beta configuration
Phenyl-beta-D-thiogalactoside	O-X replaced by S-phenyl group in the beta configuration

at 12,000 rpm in the SS1 Sorvall and the cells resuspended to the original volume in 0.1 M potassium phosphate buffer of pH 6.8.

To test for substrate utilization, the following additions were made to the main compartment of Warburg vessels. The concentrations given are those in the vessel after tipping:

methionine 1×10^{-3} M,

$MgSO_4$ 1×10^{-3} M,

sodium azide 1×10^{-2} M,

extract or whole cells of Y 665 to give a suitable rate of CO_2 evolution,

0.05 M potassium phosphate buffer of pH 6.8,

and double distilled water to make a final volume of 2 ml after tipping.

A solution of substrate to give a concentration of 0.025 M after tipping was placed in the side-arm. This concentration is above the saturation level of the enzyme; 0.1 M and higher inhibit the activity. In some experiments, 0.05 M final concentration was employed, and the results were identical to those obtained using 0.025 M substrate.

Results of galactoside hydrolysis by induced and non-induced whole cells and extracts of them are shown in Tables 7, 8, 9, and 10. Activity is expressed as micromoles substrate hydrolyzed in one minute by 1 ml of extract or cells.

The data presented in Tables 7 and 8 demonstrate that the enzyme, either in whole cells or in cell-free extracts, is specific for beta-galactosides. The rate of hydrolysis is greatest for galactosides having a cyclic structure linked to C_1 through O in the beta position. Despite the fact that in the "natural substrate", the cyclic structure would be

TABLE 7

SPECIFICITY OF YEAST BETA-GALACTOSIDASE ACTIVITY IN INDUCED WHOLE CELLS AND CELL-FREE EXTRACTS

Substrate	Rate of hydrolysis expressed as $\frac{\text{micromoles substrate}}{\text{ml x min}}$		
	extract + assay cells	whole cells + assay cells	whole cells
Lactose	54.2	4.0	0.825
ONPG	59.7	4.46	0.014*
Phenyl-beta-D-gal.	59	4.2	0.014*
Methyl-beta-D-gal.	7.4	0.6	0.254
n-butyl-beta-D-gal.	7.5	1.33	- - -
Phenyl-beta-D-thiogal.	0	0	0
Melibiose	0	0	0
Phenyl-alpha-D-gal.	0	0	0
Methyl-alpha-D-gal.	0	0	0

* Probably inhibition caused by o-nitro phenyl or phenyl group. See text for method. Final concentration of substrate, 0.025 M. Density of whole cell suspension was 100 mg per ml. Extract prepared from suspension of 100 mg wt weight per ml. Assay cells are Torula monosa for lactose, and galactose adapted S. cerevisiae, strain K, for other galactosides.

TABLE 8

SPECIFICITY OF YEAST BETA-GALACTOSIDASE ACTIVITY IN NON-INDUCED WHOLE CELLS AND CELL-FREE EXTRACTS

Substrate	Rate of hydrolysis expressed as $\frac{\text{micromoles substrate}}{\text{ml x min}}$			
	Extract + assay cells*	Whole cells + assay cells*	Whole cells	Dried cells + assay cells*
Lactose	6.4	0.22	0.14	2.78
ONPG	8.47	0.19	- - -	- - -
Phenyl-beta-D-gal.	7.54	0.17	- - -	- - -
Methyl-beta-D-gal.	0.82	0.012	0.028	0.38
n-butyl-beta-D-gal.	1.96	0.036	- - -	- - -
Phenyl-beta-D-thiogal.	0	0	- - -	- - -
Melibiose	0	0	- - -	- - -
Phenyl-alpha-D-gal.	0	0	- - -	- - -
Methyl-alpha-D-gal.	0	0	- - -	- - -

* Assay cells are Torula monosa for lactose, and S. cerevisiae, strain K, galactozymase induced, for other galactosides. See text for methods. Whole cells and dried cell values based on 100 mg wet weight per ml. Extract from suspension of 100 mg wet weight per ml. Concentration of substrate is 0.025 M.

glucose, a slightly higher rate of hydrolysis invariably occurs when ONPG or phenyl-beta-D-galactoside are substrates; that is, when a phenyl group replaces the glucose moiety. Substitution of glucose by an alkyl group results in a decreased rate of hydrolysis. Enzyme activity is greater with the n-butyl substitution than with the shorter methyl group substituent.

From Tables 7 and 8, the substrate galactosides fall into three classes according to the rates at which they are hydrolyzed, either by intact cells with assay cells, or by extracts with assay cells. Lactose, ONPG, and phenyl-beta-D-galactoside are in the first class, n-butyl-beta-D-galactoside is in the second class, and methyl-beta-D-galactoside is in the third class. This classification does not hold if the rates of hydrolysis are measured by CO_2 evolution from intact cells alone. As seen in Table 7, ONPG and phenyl-beta-D-galactoside are hydrolyzed much slower than lactose by intact cells alone. Although activity on ONPG, as measured by CO_2 evolution appeared to be almost negligible, the cup contents were bright yellow, indicating that the substrate was in fact hydrolyzed. It is therefore probable that nitrophenyl and phenyl groups inhibit the conversion of galactose to CO_2 in S. fragilis Y 665.

In Table 9 where the ratios

$$\frac{\text{micromoles lactose hydrolyzed/(ml x min)}}{\text{micromoles designated substrate hydrolyzed/(ml x min)}}$$

are calculated, the three classes of substrate galactosides are again seen. When assay cells are employed in the measurement, the ratio has a value of 1 for compounds of class 1, 3 for compounds of class 2, and 7 for compounds of class 3. A second difference between compounds of class 1 and classes 2 and 3 is seen when the ratios from intact cells plus assay cells, and

TABLE 9

RATIOS OF $\frac{\text{MICROMOLES LACTOSE HYDROLYZED / (ML x MIN)}}{\text{MICROMOLES DESIGNATED SUBSTRATE / (ML x MIN)}}$

Substrate	Cells	Extract + assay cells	Whole cells + assay cells	Whole cells
Lactose	induced	1.0	1.0	1.0
	non-induced	1.0	1.0	1.0
ONPG	induced	0.91	0.9	58.5
	non-induced	0.76	1.09	---
Phenyl-beta-D-gal.	induced	0.92	0.96	5.95
	non-induced	0.85	1.16	---
Methyl-beta-D-gal.	induced	7.35	6.75	3.1
	non-induced	7.86	17	4.45
n-butyl-beta-D-gal.	induced	3.02	3.0	---
	non-induced	3.48	5.65	---

Ratios calculated from the data in tables 7 and 8.

cell-free extracts plus assay cells are considered. Compounds of class 1 have the same ratio when measured under these four conditions (i.e. induced and constitutive intact cells and extracts from them). However, for compounds of classes 2 and 3, the ratio is twice as great when measured with intact non-induced cells plus assay cells as under any of the other conditions.

A measurement of crypticity is given by the ratios

$$\frac{\mu \text{M substrate hydrolyzed/(ml x min), intact cells + assay cells}}{\mu \text{ M substrate hydrolyzed/(ml x min), extract + assay cells}}$$

calculated in the third column of Table 10. It can be seen that the crypticity of non-induced cells is greater than that of induced cells on all substrates. The factor decrease of crypticity is greatest for compounds of classes 2 and 3.

It is evident from column 5 of Table 10 that the beta-galactosidase in intact cells hydrolyzes its substrate faster than the products of the reaction can be utilized. Furthermore, the excess products become extracellular.

The rates of CO_2 evolution from lactose and methyl-beta-D-galactoside are compared with the rates from glucose and galactose in Table 12. The induced cells utilize both galactosides at a greater rate than the constitutent hexoses, whereas the non-induced cells utilize the galactosides at a rate approximately equal to glucose and galactose. These results can be interpreted to indicate that in induced cells either the disaccharide or its hydrolysis products can penetrate the cell more readily than galactose or glucose, that the hydrolysis products are not glucose and/or galactose, but a compound more readily metabolized by the cell, or that there is a

TABLE 10

COMPARISON OF SUBSTRATE HYDROLYSIS BY EXTRACTS AND WHOLE CELLS ON THE BASIS OF RATIOS OF MICROMOLES SUBSTRATE HYDROLYZED / (ML x MIN)

Substrate	Cells	Extract+a.c.* / whole cells+a.c.	Extract+a.c.* / whole cells	Whole cells+a.c.* / whole cells
Lactose	induced	13.5	65.7	5
	non-induced	31.4	46	1.47
ONPG	induced	12.3	4220	316
	non-induced	44	-----	-----
Phenyl-beta-D-gal.	induced	14.1	423	30
	non-induced	43	-----	-----
Methyl-beta-D-gal.	induced	12.4	27.8	2.24
	non-induced	68	-----	0.42
n-butyl-beta-D-gal.	induced	13.5	-----	-----
	non-induced	61	-----	-----

Ratios calculated from the data in Tables 7 and 8.

* a.c.-abbreviation for assay cells, either Torula monosa, or galactose adapted S. cerevisiae, strain K.

"direct utilization" of lactose by the cell (15,16,17,18,19).

It should be noted that freshly prepared crude extracts have activity on methyl-beta-D-thiogalactoside (TMG). However, this activity is short-lived, and cannot be attributed to the yeast beta-galactosidase (see Table 11).

These data clearly show that in S. fragilis, strain Y 665, the induction process causes three basic changes in the cells.

(i) The rate of beta-galactosidase synthesis is increased. Cells for the extracts were harvested at the same O.D., and resuspended to the same concentration. Yet extracts of induced cells hydrolyze each substrate ten times faster than extracts of non-induced cells.

(ii) The crypticity factor is decreased. Therefore, intact induced cells have a greater relative rate of substrate uptake than non-induced cells.

(iii) On the basis of ratios of the rates of substrate hydrolysis (Table 9), extracts of beta-galactosidases from induced and constitutive cells are indistinguishable.

B. Synthesis of Beta-galactosidase in Growing Cells

The beta-galactosidase in S. fragilis is particularly suited to a comparative study of the kinetics of constitutive and induced enzyme formation. Because the inducible cell synthesizes levels of the constitutive enzyme which can be accurately assayed, the kinetics of the formation of each enzyme can be studied against a constant genetic background. It is, therefore, useful to compare the results of such experiments with those from the beta-galactosidase in E. coli, where a constitutive mutant of the inducible strain was employed.

TABLE 11

STABILITY OF ENZYME ACTIVITY ON LACTOSE, METHYL-BETA-D-GALACTOSIDE, AND METHYL-BETA-D-THIOGALACTOSIDE (TMG)

Enzyme preparation	Enzyme activity* on			Ratio $\frac{\text{Activity* lactose}}{\text{Activity* } CH_3\text{-}\beta\text{-D-gal.}}$	Ratio $\frac{\text{Activity* lactose}}{\text{Activity* TMG}}$
	Lactose	CH_3-β-D-gal.	TMG		
original	54.3	7.4	4.85	7.35	11.2
22 hrs 0°C	54	6.2	0.6	8.8	90
22 hrs 5°C	49.2	5.72	0.55	8.6	89

* Activity expressed in $\frac{\text{micromoles substrate hydrolyzed}}{\text{ml x min}}$

Cell-free extract was prepared from induced S. fragilis, Y665, and tested manometrically for activity on the three substrates immediately. Following storage at 0°C and 5°C for 22 hours, the activity in the extracts on the three galactosides was measured.

TABLE 12

RATE OF FERMENTATION OF GALACTOSIDES, GLUCOSE, AND GALACTOSE BY S. FRAGILIS WHOLE CELLS

Cells		Micromoles substrate metabolized / ml x min				
		galactose	glucose	galactose + glucose	lactose	β-CH_3-D-gal.
Induced		0.03	0.07	0.056	0.344	0.11
Non-induced	exp. 1	0	0.032	0.039	0.045	0.005
	exp. 2	0.015	0.07	0.063	0.058	0.012

1. Experimental procedure

Unpublished experiments by Spiegelman have demonstrated galactose to be an inducer for beta-galactosidase activity in S. fragilis. This compound was employed for the majority of the experiments on the induction phenomenon.

Induction experiments in growing cells followed the general procedure to be outlined. Experimental cultures were inoculated and incubated as previously described, until an O.D. ranging from 0.05 to 0.1 was attained. 13.5 ml of such cells were dispensed into a calibrated 20 cm x 2.5 cm tube. For induction purposes, an addition of galactose to a final concentration of 0.5 % (approximately 0.03 M) was made. The final volume in all tubes was made up to 15 ml with medium. O.D. measurements and samples for enzyme assay were immediately taken. The tubes were then incubated as noted in Methods, and at intervals during the incubation period, optical density measurements were made simultaneously with the removal of samples for enzyme assay. The samples were immediately treated with A.R. benzene to destroy the crypticity (see Section 3 below), and stored at $0^{\circ}C$ until activity was measured by the ONPG technique. No significant loss of enzyme occurred over three days of storage under such conditions.

2. Synthetic medium for growth and enzyme synthesis

Each of the three synthetic media was tested for beta-galactosidase synthesis during growth of S. fragilis. 1 % glycerol and 1 % ethanol were employed as carbon source, and the experiments performed both in the presence and absence of 0.25 % amino acids. It was immediately evident that the modified Davies medium was the most satisfactory, not only for enzyme synthesis, but also for the faster growth rate it permitted. Ethanol was the superior carbon source. Growth rate was slower in glycerol, and

constitutive enzyme formation was suppressed. Induced enzyme synthesis was similar with both carbon sources. Therefore, the Davies medium, with 1 % ethanol as carbon source was chosen for subsequent experiments.

3. Treatment of cells to destroy crypticity of the enzyme

Because the yeast beta-galactosidase was cryptic, and difficult to measure in intact cells, it was essential to find a simple technique which would allow full enzyme expression. Treatment with toluene (37), ethyl acetate (37), benzene (38), repeated freezing and thawing (39), sonic oscillation, and isoamyl alcohol (40) were tested. As seen from Table 13, benzene treatment was superior to, or as good as any of the other procedures employed. Toluene, ethyl acetate, and benzene treatment were examined for reliability of enzyme expression in growing cultures, by assaying for enzyme during the growth and induction cycles. Addition of one-tenth volume benzene to the cell sample, followed by shaking at 30°C for 20 minutes proved to give the most satisfactory results, and was the procedure routinely followed in the experiments described hereafter.

4. Kinetics of beta-galactosidase synthesis in S. fragilis

a. Effect of inducer on kinetics of enzyme synthesis. Although it has been found that 80-100 molecules of penicillin per cell are adequate to induce penicillinase in B. cereus, (41), the lowest level of inducer for the beta-galactosidase in E. coli approximates 1 x 10^{10} molecules per cell (42, 43). This range of inducer concentrations is indicative that for each induced enzyme system, there is a level of inducer optimum for induction of its enzyme. For this reason, experiments were performed to determine the level of inducer required for optimum induction of the beta-galactosidase in S. fragilis. Because a non-utilizable inducer for this system had not been found, galactose was employed for these experiments; it was a known

TABLE 13

ENZYME ACTIVITY OF CELLS TREATED WITH VARIOUS AGENTS TO DESTROY THE CRYPTICITY OF THE BETA-GALACTOSIDASE

Cells grown in Davies medium plus:	O.D. of cells	$\frac{m\mu M \text{ ONPG hydrolyzed}}{ml \times min}$ by cells treated with agents:						
		Toluene	E.A.A.	Benzene	Iso-amyl alcohol	Sonication	Freezing +thawing	0
Ethanol	1.7	46.8	72	92.5	78	63.2	37.7	5.15
Ethanol + galactose	1.3	214	272	320	264	257	178	----
Ethanol + AA	2.64	42.9	160	191	136	31.9	112	3.46
Ethanol + AA + galactose	2.64	220	358	548	418	140	342	30.6

Enzyme activity was measured by the ONPG technique. The cells in all cases excepting ethanol-galactose grown were in stationary phase. The ethanol-galactose grown cells were in late log phase. Toluene, E.A.A. (ethyl acetate), benzene and iso-amyl alcohol treatments were performed by the addition of one-tenth volume of the specified agent to 1 ml cells, and shaking at 30°C for 40 minutes. Rapid freezing and thawing procedures were repeated 5 times. Sonication of 15 ml volumes was performed as previously described.

inducer of beta-galactosidase in S. fragilis (28), and its rate of utilization by whole cells was lower than that of the beta-D-galactosides.

Induction during growth was tested with final inducer concentrations of 0.5 M, 0.3 M, 0.1 M, 0.08 M, 0.06 M, 0.04 M, 0.02 M, 0.01 M, and 0 M. The cultures were grown in Davies medium supplemented with 1 % ethanol and 0.125 % enzyme hydrolyzed casein.

Graphs of enzyme activity vs. mass (E vs M) at representative galactose concentrations are shown in Figures 8a and 8b. Several interesting features emerge on comparing the results obtained at different inducer levels. It will be noted that both enzyme synthesis and growth are inhibited by galactose concentrations of 0.3 M and 0.5 M. At inducer levels of 0.1 M and lower, the curve consists of two parts; a linear region, followed by one which appears to be exponential. The latter region is shown to be a true exponential increase in enzyme with respect to mass in the semi-logarithmic plots 9a and 9b. Henceforth, direct proportionality between enzyme synthesis and mass formation will be termed linear enzyme synthesis. On the other hand, the linear region found in the semi-log plots in Figures 9a and 9b will be designated exponential enzyme synthesis.

In Figures 8a and 8b, and 9a and 9b, it is seen that the inducer concentration affects the slopes of both linear enzyme and exponential enzyme syntheses. In both instances, the slopes increase with decreasing levels of galactose. Also, the length of each region of enzyme formation appears to increase with decreasing inducer levels. Thus, above 0.04 M inducer, the onset of exponential synthesis of enzyme is delayed with increasing levels of galactose. At levels above 0.3 M, the exponential region does not occur.

The above results show that both linear and exponential synthesis of beta-galactosidase can occur sequentially in the same culture of S. fragilis. It is therefore pertinent to enquire if a correlation can be

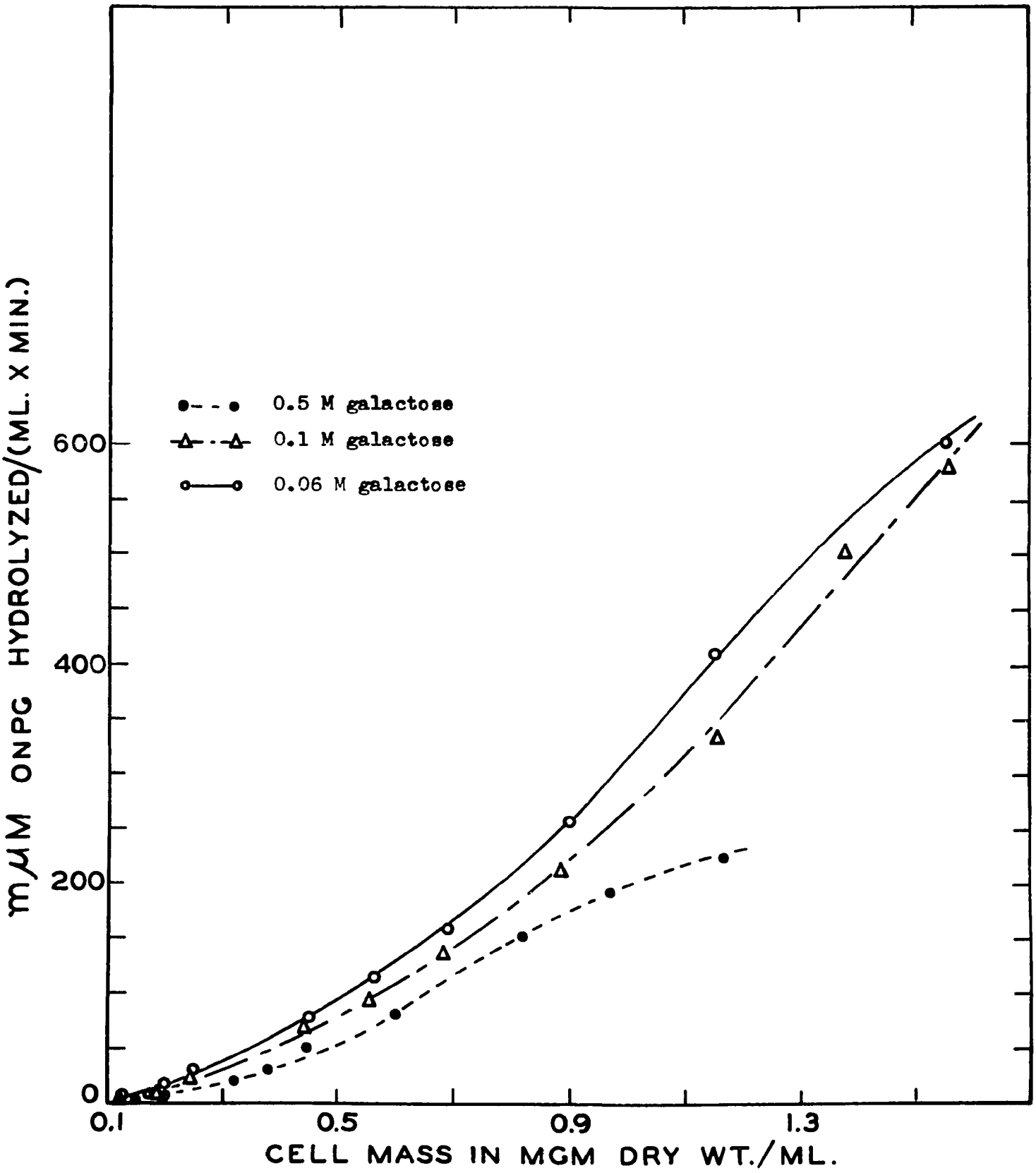


Fig. 8a. Effect of galactose concentration on beta-galactosidase synthesis by S.fragilis.

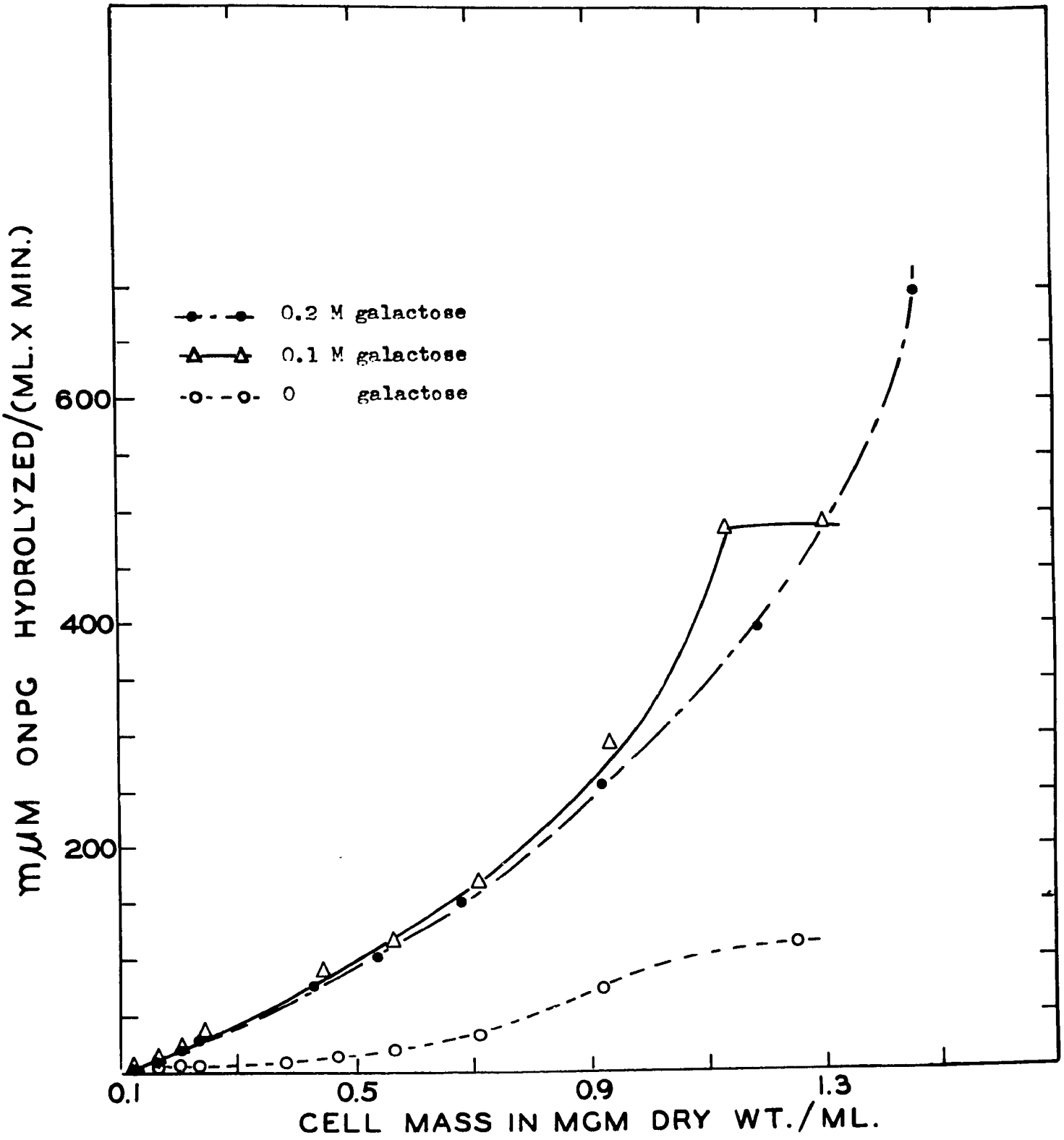


Fig. 8b. Effect of galactose concentration on beta-galactosidase synthesis by S. fragilis.

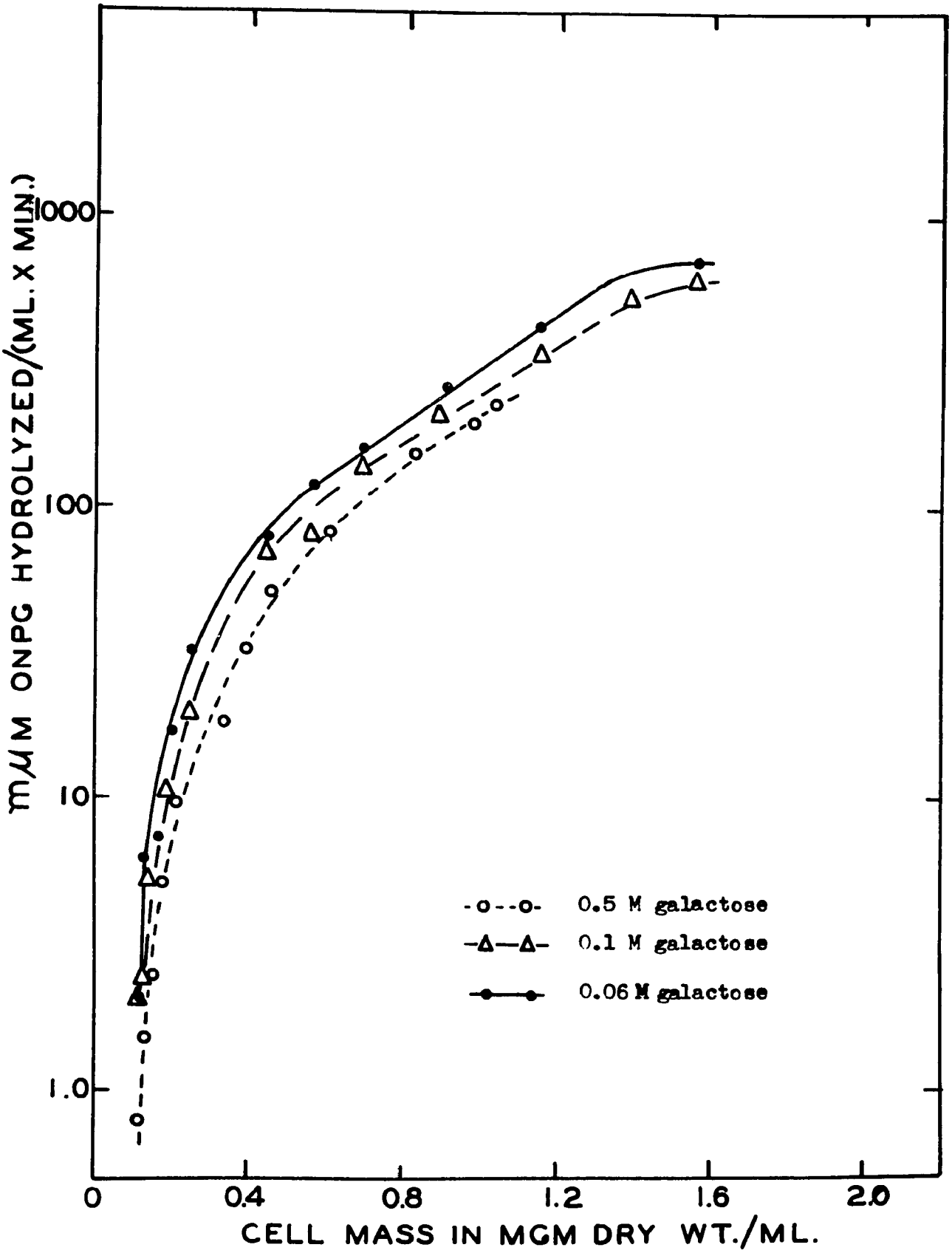


Fig. 9a. Effect of galactose concentration on beta-galactosidase synthesis in S. fragilis.

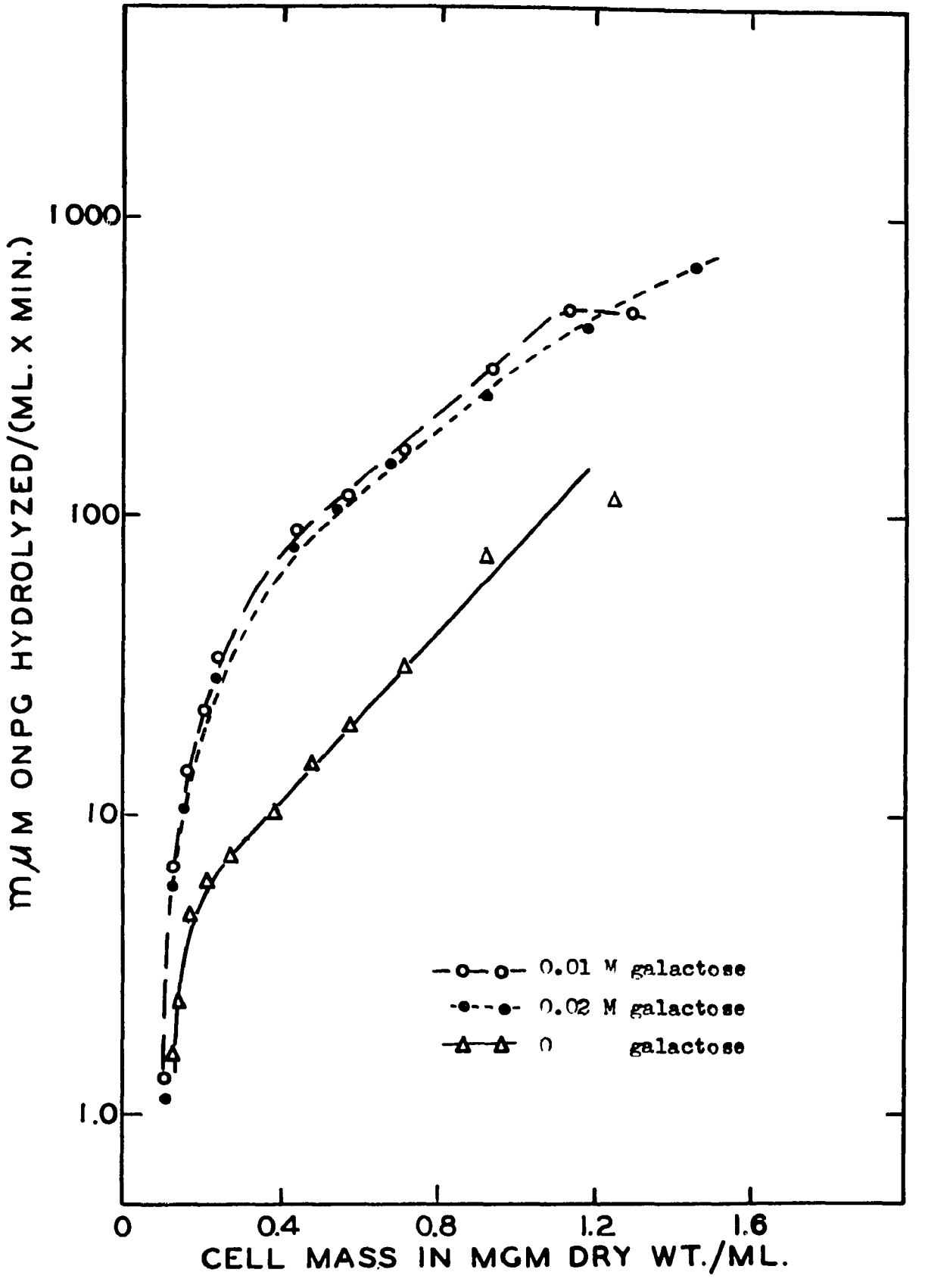


Fig. 9b. Effect of galactose concentration on beta-galactosidase synthesis in S. fragilis.

established between the growth of the culture, and the transition from linear kinetics to exponential kinetics. For this purpose, a comparison should be made of Figures 8a and 8b, and 9a and 9b, with the corresponding growth curves in Figures 10a and 10b. It will be noted that the linear enzyme synthesis obtains only during the period of logarithmic increase in mass. The length of the region of linear enzyme synthesis is therefore a reflection of the growth of the population. The transition from linear kinetics to exponential kinetics is found to occur at the end of the exponential growth phase, providing that inducer concentrations are held below 0.04 M.

Since the exponential increase of enzyme with mass occurs as the growth rate falls, it would appear that a decreased growth rate is selectively advantageous for beta-galactosidase synthesis. One might then expect that the relative rate of enzyme synthesis, dE/dM, would increase with generation time, g. Figure 11 shows that this is indeed the case. From Figure 11, it is also evident that at a given generation time, the value of dE/dM decreases with increasing inducer concentration. Therefore, the inhibition of enzyme formation by increasing levels of inducer cannot be attributed to effects on the growth rate, but rather is a true inhibition of the beta-galactosidase synthesis.

b. Kinetics of constitutive enzyme synthesis. Excepting quantitative differences in the slopes, the kinetics described above for induced beta-galactosidase synthesis are also found for constitutive beta-galactosidase synthesis. They may be summarized as follows:

(i) A comparison of Figures 8b and 10b shows that $dE/dM = k$, when g is constant (i.e. during exponential growth).

(ii) Figure 11 demonstrates that dE/dM varies with g.

(iii) From Figures 9b and 10b, it is seen that E increases exponentially with mass at the end of the exponential

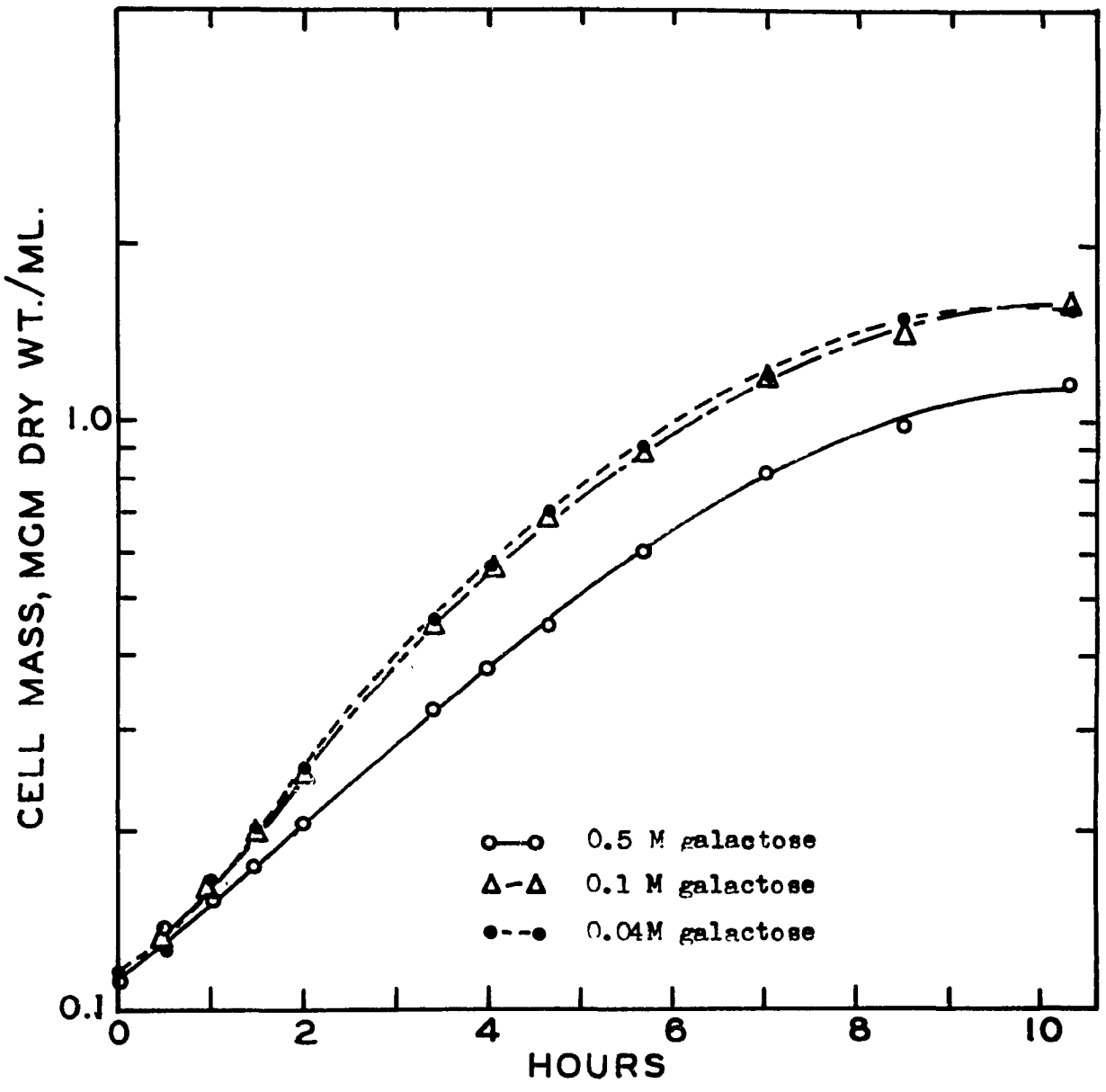


Fig. 10a. Effect of galactose concentration on growth of S. fragilis. The points on each curve correspond to points on the curves in figures 8a and 9a.

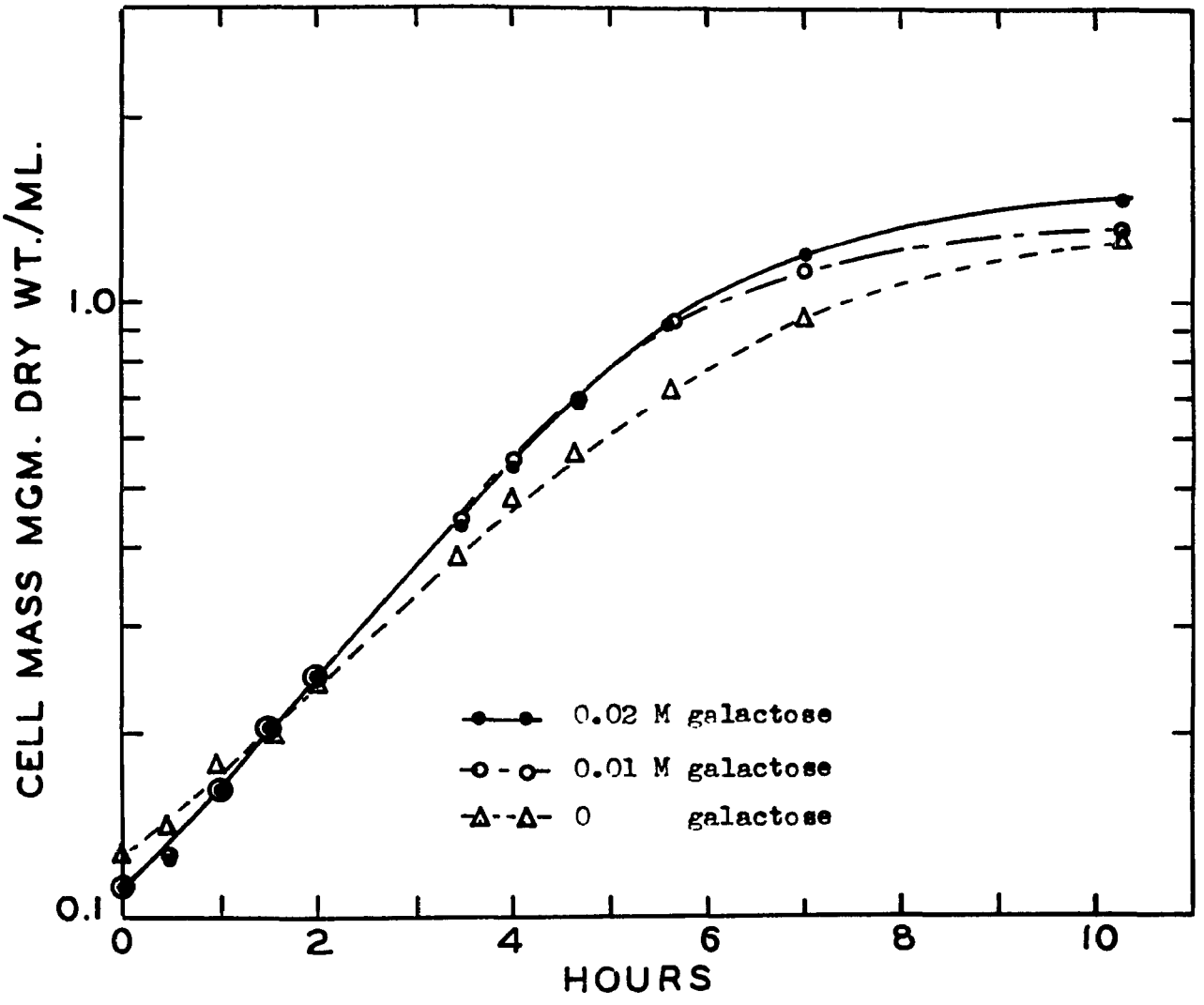


Fig. 10b. Effect of galactose concentration on growth of S. fragilis. The points on each curve correspond to points on the curves in figures 8b and 9b.

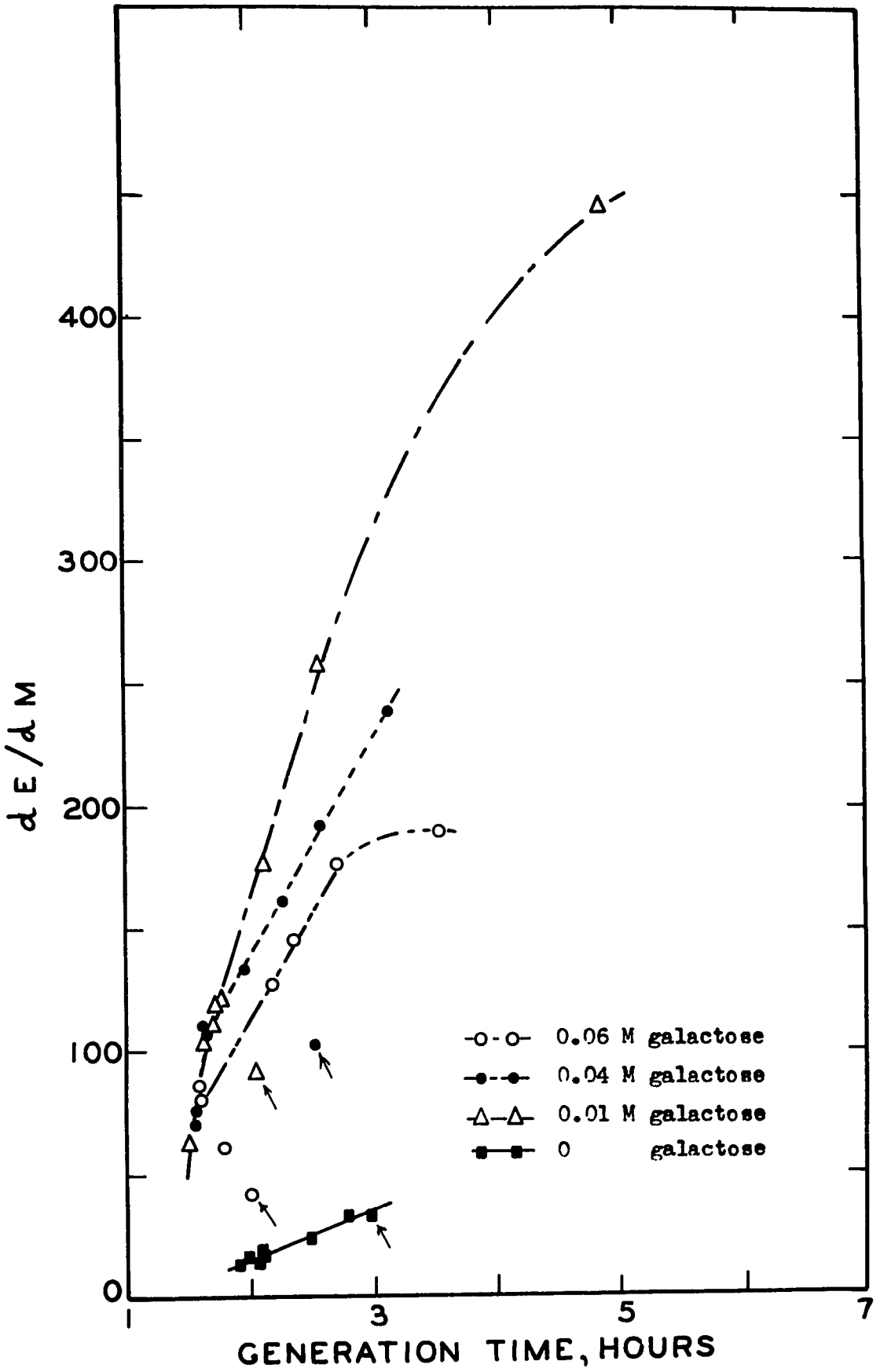


Fig. 11. Effect of galactose concentration on relation between enzyme synthesized per unit mass and generation time. Arrow denotes dE/dM value during initial lag of growth.

growth phase.

These data clearly show an interaction to exist between the relative rate of enzyme synthesized, dE/dM, and the generation time.

c. Effect of amino acids, nucleosides, and purines and pyrimidines on the kinetics of beta-galactosidase synthesis. An obvious explanation for an increase in enzyme synthesis concomitant with a decrease in generation time is that a substance present in limiting quantities is required for both mass and enzyme formation. On the basis of this hypothesis, experiments were performed in an attempt to define the nature of this substance.

One candidate for such a material would be amino acids. Because the experiments described above were performed in 0.125 % amino acids, the effect of amino acids was studied at levels of 0 % and 0.25 %. Davies medium, 1 % in ethanol, was supplemented with 0.5 % (0.03 M) galactose for induction experiments. The results of a typical experiment are shown in Figures 12, 13, 14, and 15. Except for quantitative differences in the slopes, induced and constitutive enzyme syntheses under these conditions exhibit kinetics similar to those described above in 0.125 % amino acids. Therefore, amino acids are not the limiting substance in question, although they do enhance the growth rate.

There is, however, one point of particular interest to be found in these experiments. In the plot E vs M (Figure 12), amino acids appear to inhibit both constitutive and induced enzyme syntheses. Yet, at comparable values of g in Figure 15, the value of dE/dM is greater in the presence of amino acids than in their absence. Over the same cell density change, cells growing in amino acids exhibit a lower g than cells in the absence of amino acids; e.g. for cell densities ranging from approximately 0.3 to 0.4, induced cells in the absence of amino acids have a g of 25,

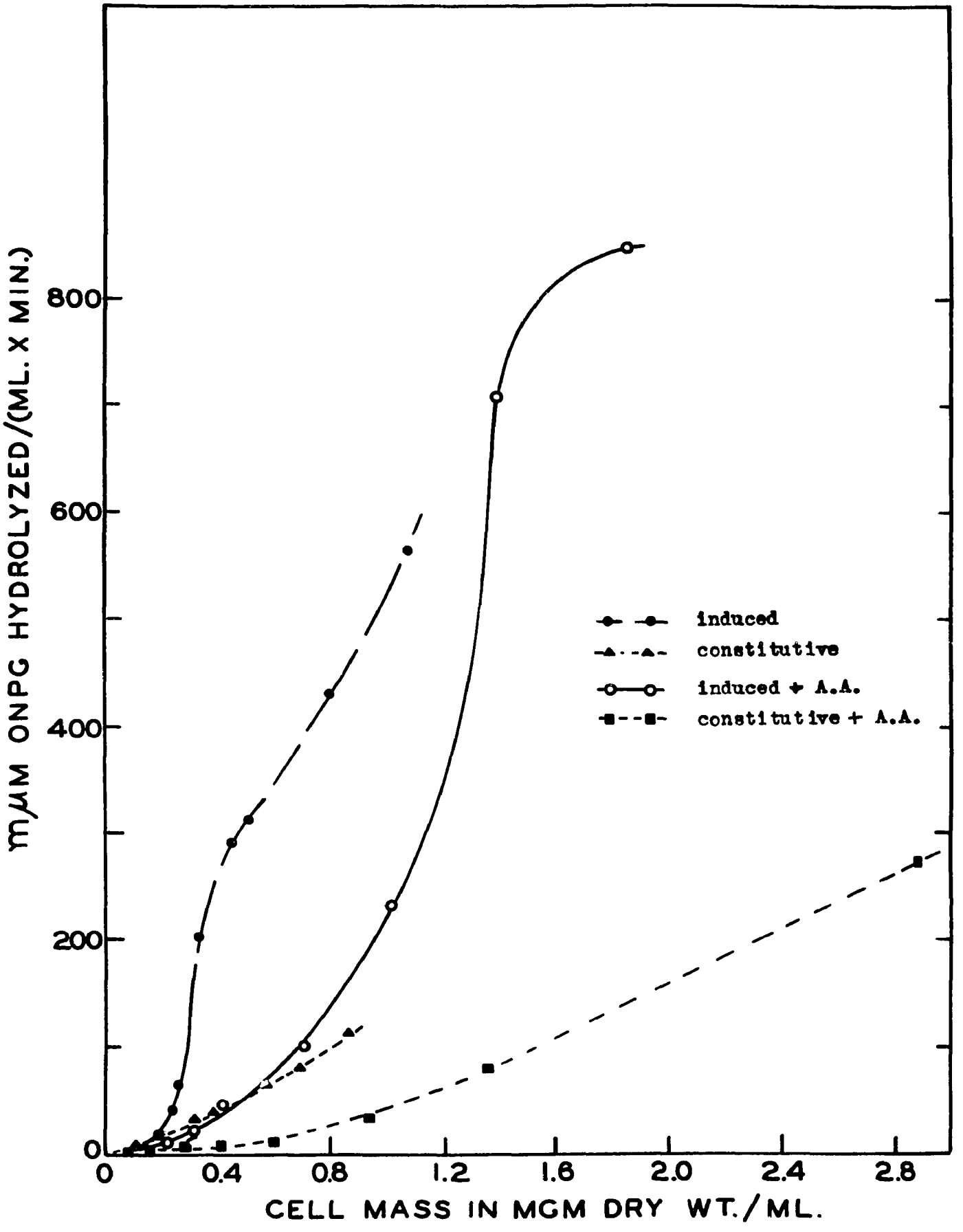


Fig. 12. Effect of amino acids on beta-galactosidase synthesis in S. fragilis.

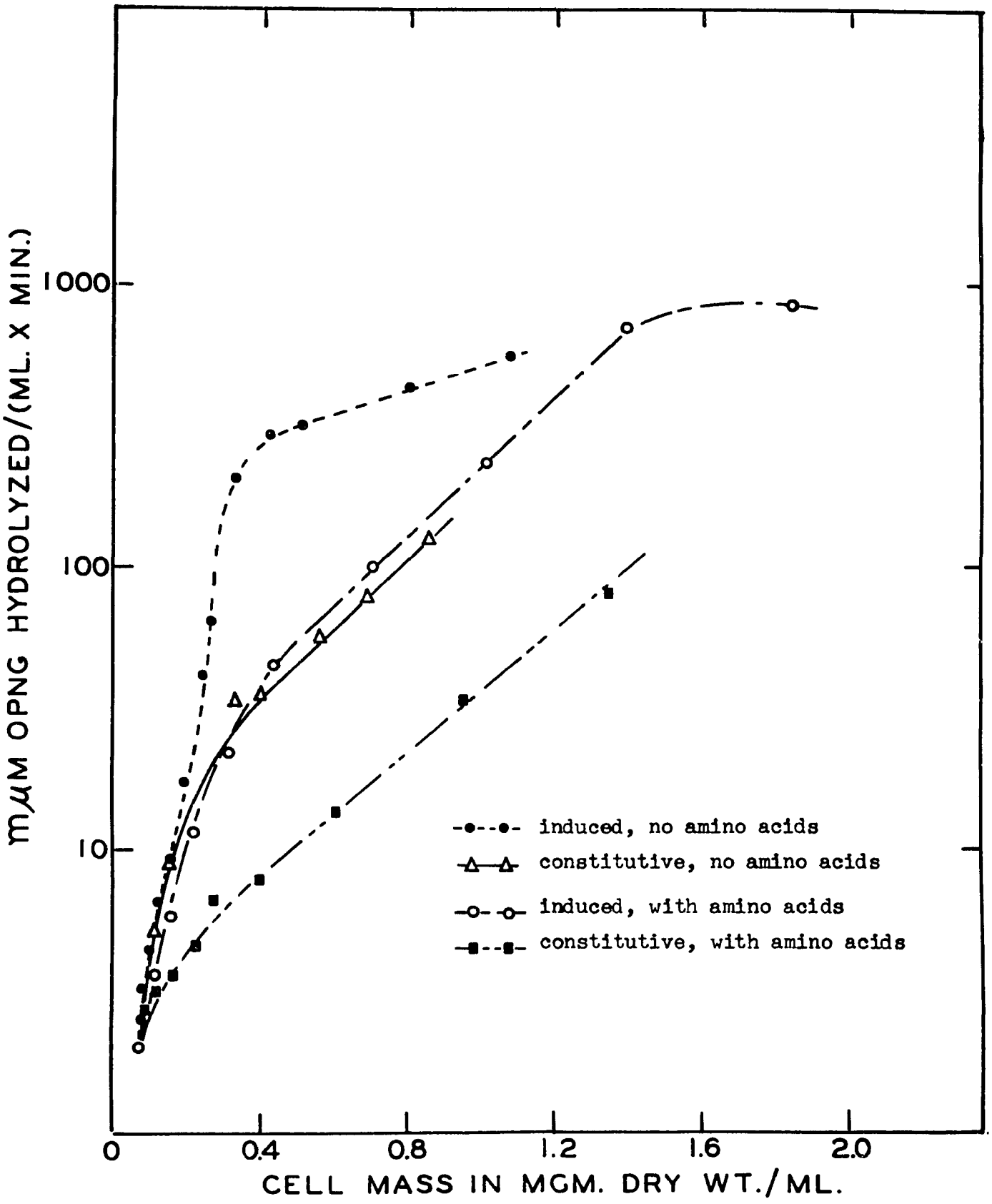


Fig. 13. Effect of amino acids on beta-galactosidase synthesis in S. fragilis.

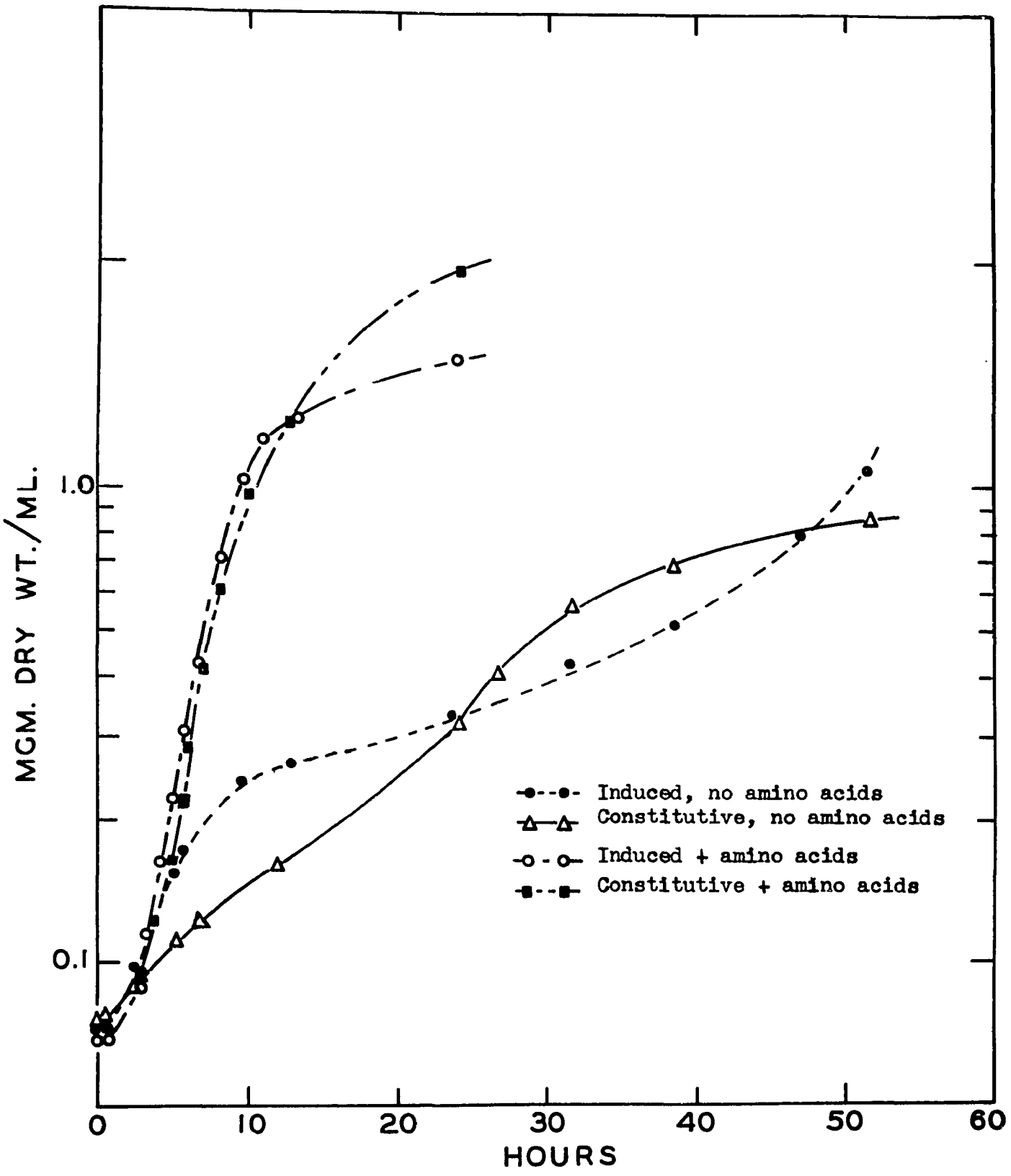


Fig. 14. Effect of amino acids and galactose on growth of S. fragilis. The points on each curve correspond to the points on corresponding curves in figures 12 and 13.

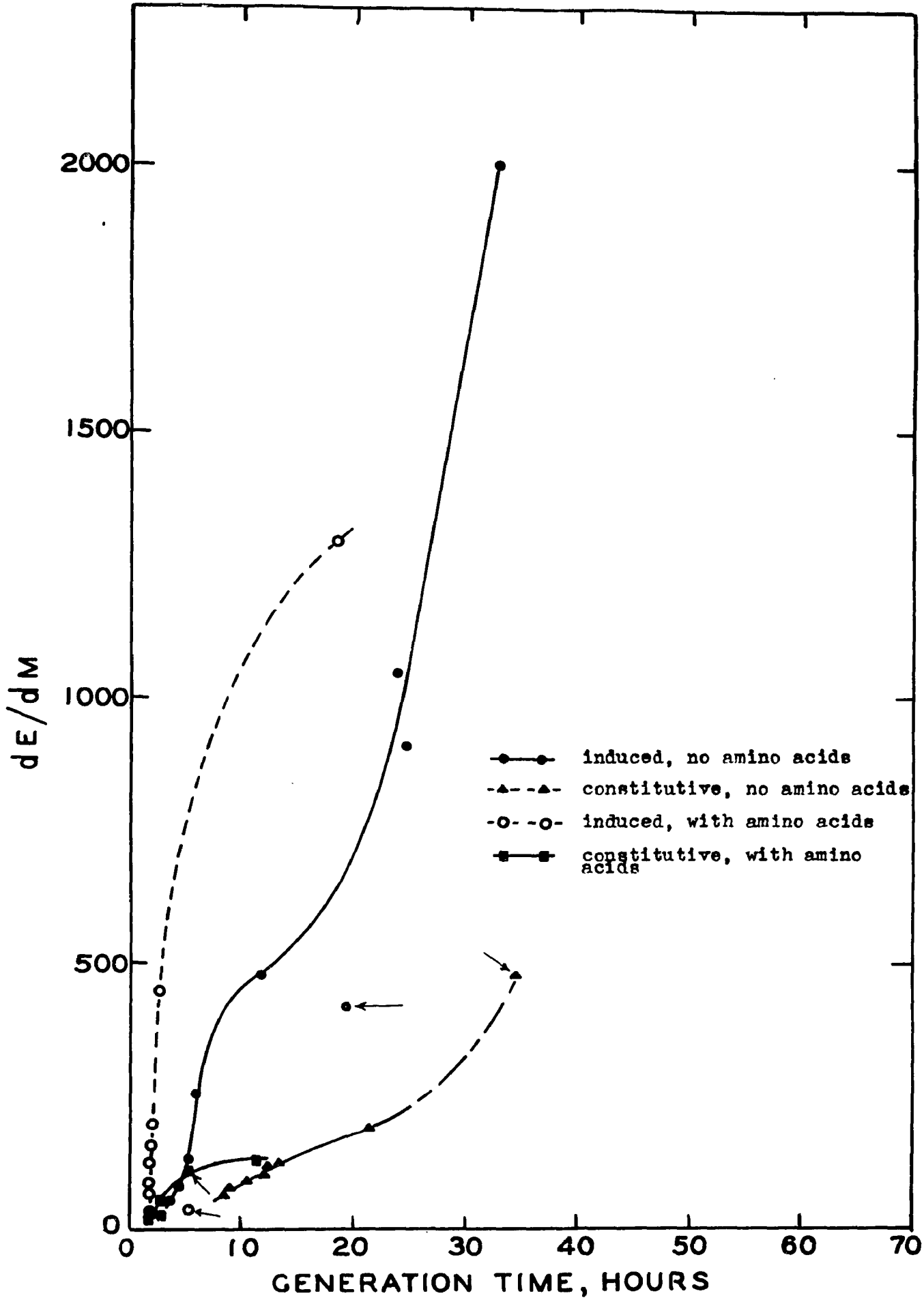


Fig. 15. Relation between enzyme synthesized per unit mass and generation time. Arrow denotes dE/dM value during initial lag of growth.

whereas in the presence of amino acids, the g is 2. The apparent inhibition of enzyme synthesis by amino acids is probably caused by a low g resulting in dE/dM values lower than those in the absence of amino acids.

Further attempts to define the postulated mutually required substance were made by supplementing the growth tube of both induced and constitutively grown cells with the following compounds:

(i) a mixture of purines and pyrimidines, each at a level of 100 micrograms per ml,

(ii) ribose at a level of 0.2 mgm per ml, and

(iii) a mixture of nucleosides, each at a concentration of 100 micrograms per ml.

Cultures were made in 1 % ethanol Davies medium with and without 0.25 % amino acids, and the experiments performed as previously described. There was no significant effect on enzyme formation under any of the conditions tested. Therefore, if the interaction between growth and enzyme formation is caused by limiting concentrations of a mutually required substance, the nature of this substance remains undefined.

d. Cell age and the relationship between dE/dM and g. The relationship between dE/dM and g is of particular interest in an understanding of enzyme synthesis if the phenomenon is not solely attributable to cell age. There are a number of experimental results which demonstrate that cell age is not the determining factor.

(i) The nature of the growth curves of cells grown in the absence of amino acids (see Figure 14) enables dE/dM to be determined at values of g which recur non-sequentially in the growth. Results of such analyses show that for every g, there is a unique value of dE/dM.

(ii) Because the cells are grown in a large flask, and transferred to tubes at the beginning of the experiment, there is usually a period of slower growth before the exponential phase. With constitutively grown cells, this lag is useful for examining dE/dM at the same g during different parts of the growth curve. Where the g found during this initial lag occurs later in the growth of the culture, the two values of dE/dM are the same.

(iii) The generation times of S. fragilis, strain Y 665, the hybrid, FPR-1, and S. cerevisae, strain K are decreased with decreasing ethanol concentration. For example, the data of experiments presented in Figure 16 show for induced cells of densities between 0.3 and 0.39, a g of 42 in 1 % ethanol and a g of 4.7 in 0.5 % ethanol. For non-induced cells between densities 0.35 to 0.58, the g is 9 in 1 % ethanol, and 4 in 0.5 % ethanol. Thus the relative rates of enzyme synthesis can be studied in two cultures growing at different rates.

Data from a typical experiment are presented in Figures 16 and 17. In an E vs M plot (Figure 16) both induced and constitutive beta-galactosidase syntheses appear to be inhibited in 0.5 % ethanol. However, at a given g in Figure 17, the relative rate of enzyme synthesis in 0.5 % ethanol equals or slightly exceeds that in 1 % ethanol. The inhibition seen in the E vs M plot is caused by the fact that cultures at the same density are growing at different rates, and have correspondingly different dE/dM values. Similar experiments performed in the presence of amino acids give the same results. The phenomenon has also been observed for induction of galactozymase in

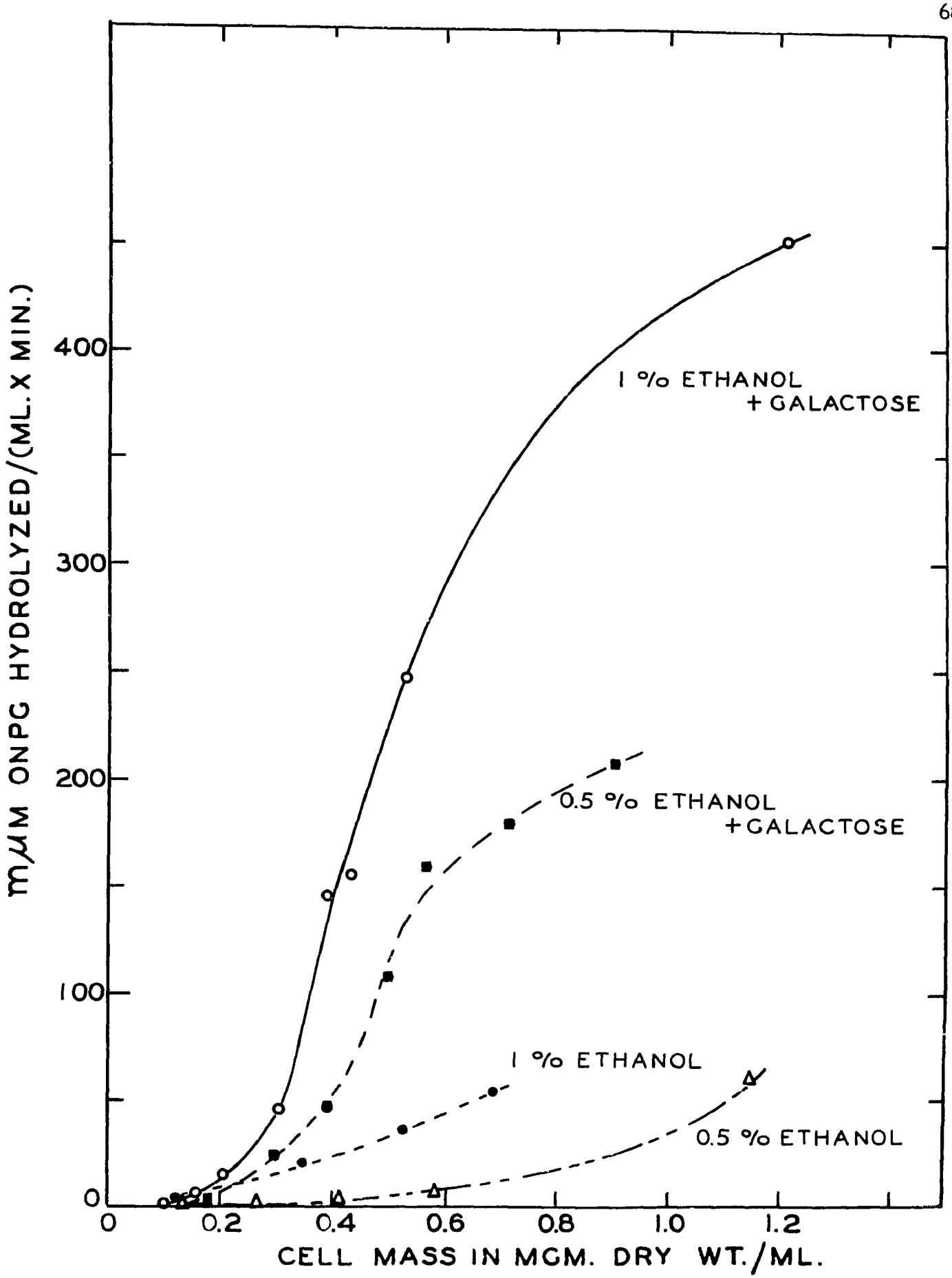


Fig. 16. Effect of ethanol concentration on the synthesis of induced and constitutive beta-galactosidase. Cell mass in mgm dry wt/ml.

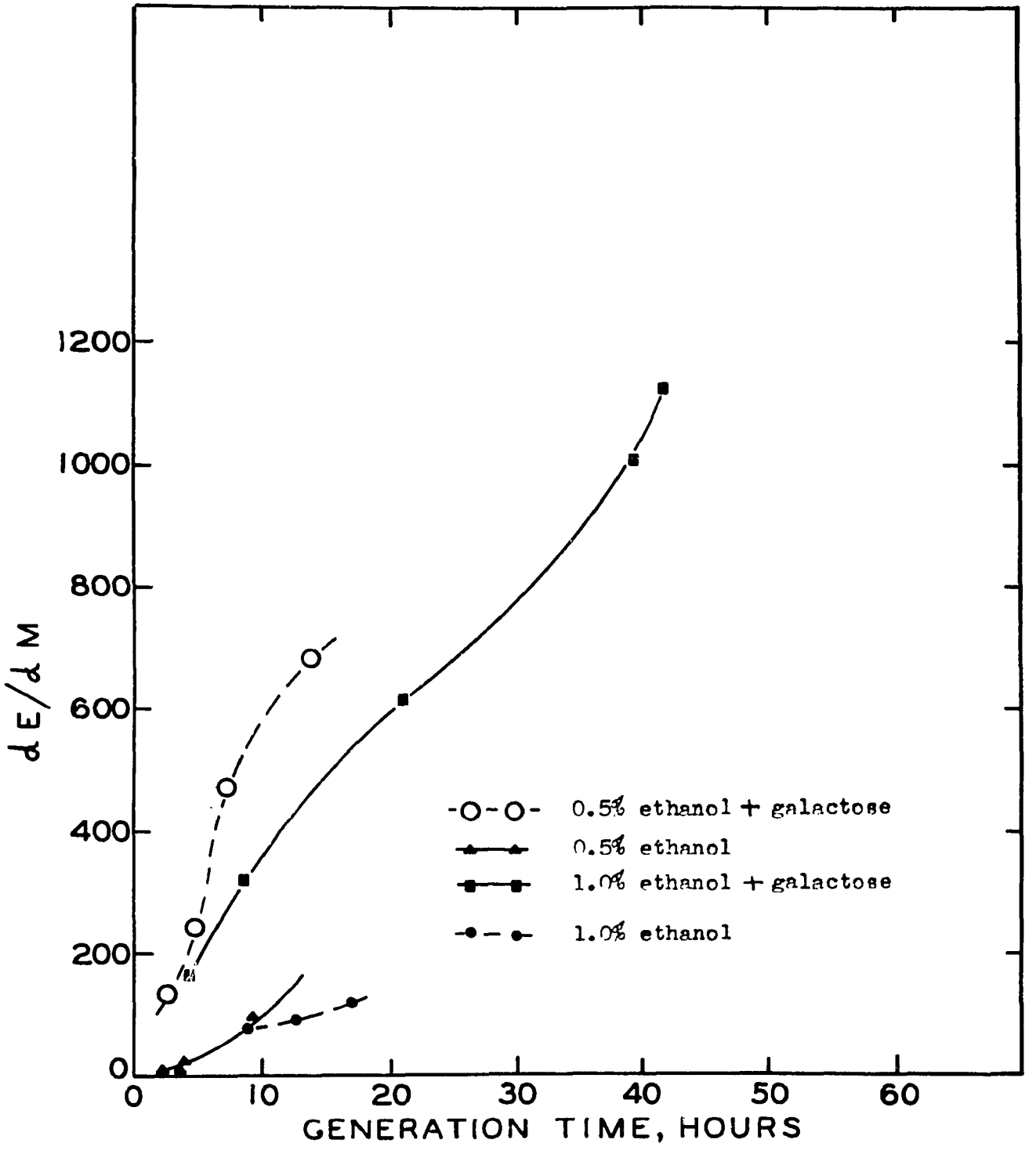


Fig. 17. Effect of ethanol concentration on relation between enzyme synthesized per unit mass and generation time. Calculated from data in figure 14.

S. cerevisae, strain K, and alpha-glucosidase in the hybrid, FPR-1.

Thus, an increase of dE/dM with g is not a reflection of cell age, but is indeed a true interaction between the rate of mass formation and the rate of enzyme synthesis.

e. Rate of mass formation and relative rate of enzyme formation during exponential synthesis of beta-galactosidase. During the exponential synthesis of enzyme, where $dE/dM = cE$, dE/dM will be a function of c and E. Therefore it is of interest to compare the values of c for constitutive and induced synthesis of enzyme. An examination of the slopes of the exponential region in Figure 13 shows that:

(i) in the presence of 0.25 % amino acids, c is the same for induced and constitutive cells, whereas,

(ii) in the absence of amino acids, c is not the same for induced and constitutive cells.

It is therefore of considerable importance to determine the reason for the difference between (i) and (ii) above.

The following results are found on inspection of the average growth rate during this region.

(i) In the presence of 0.25 % amino acids, constitutive and induced cells exhibit the same average growth rate (see Table 14).

(ii) In the absence of amino acids, induced cells have a c of 0.32, and a growth rate twice as great as that of non-induced cells which have a c of 1.0 (see Table 14).

It therefore appears that c increases with a decrease in growth rate. Because of this conclusion, a critical comparison of c in induced

TABLE 14

COMPARISON OF SLOPE AND AVERAGE MGM MASS FORMED PER HOUR

WHEN LOG E IS PROPORTIONAL TO M

Exp. #	Constitutive, no amino acids		Induced, no amino acids		Constitutive, with amino acids		Induced, with amino acids	
	$\frac{\text{av mgm mass}}{\text{ml x hr}}$	slope	$\frac{\text{av mgm mass}}{\text{ml x hr}}$	slope	$\frac{\text{av mgm mass}}{\text{ml x hr}}$	slope	$\frac{\text{av mgm mass}}{\text{ml x hr}}$	slope
48	0.0282	1.04	0.042	0.325	0.1	0.53	0.154	0.71
45	0.015	1.0	0.025	0.33	0.167	0.78	0.152	0.97
	0.016	1.0	0.03	0.29	0.163	0.78	0.142	0.97
	0.021	1.0	0.032	0.29	0.158	0.78	0.132	0.97
49	0.03	1.04	0.017	0.32	0.19	0.7	0.193	0.75
	0.023	0.933	0.016	0.36	0.173	0.7	0.197	0.7
105 (0.125 % amino acids)					0.136	1.13	0.184	0.84

and non-induced cells can only be made when the average growth rates of the two types of cells are the same, i.e., in the presence of 0.25 % amino acids. Since induced and non-induced cells exhibit the same c when the average growth rates are the same, it appears that the same limiting factors are involved for the synthesis of induced and constitutive beta-galactosidase in region 2.

f. Initial lag of induced enzyme synthesis. As previously noted, because of the manner in which the experiments have been performed, there is initially a variation in g. Because dE/dM varies with g, it is impossible to examine an E vs. M plot in any simple manner for a lag in enzyme formation. However, should such a lag exist, the values of dE/dM should be lower than those at the same g later in the experiment. On the basis of such an analysis, a lag has been found. It lasts no longer than 30 minutes in the presence of amino acids, and no longer than two hours in their absence.

5. Effect of glucose on beta-galactosidase synthesis in S . fragilis

Because glucose has often been reported to inhibit induced enzyme synthesis, it was of interest to test the effect of glucose on the system herein reported. Of particular interest, however, was a comparison of its effect on constitutive and induced beta-galactosidase synthesis in E. coli and S. fragilis. In the former organism, Monod and Cohn have reported that

(i) the simultaneous addition of glucose and inducer prevents induced beta-galactosidase synthesis (44),

(ii) the addition of glucose after induction has begun results in a lowering of the slope in an E vs M plot (44), and

(iii) glucose affects a decreased slope in a plot of constitutive enzyme formation vs mass (45).

Two types of cultures were employed in this experiment: cells grown in Davies medium with 1 % ethanol and 1 % glucose to test the effect of glucose, and cells grown in 1 % ethanol for controls. Galactose at 0.03 M was used for the inducer. Where the effect of amino acids was tested, they were supplied at 0.25 % concentration. In the experiments reported here, 2 % more glucose was added at the beginning of the experiment in order to prevent glucose exhaustion.

Data from a typical experiment are given in the E vs M graphs of Figures 18 and 19. The results can be summarized as follows:

(i) under all conditions, there is an initial decrease of total enzyme in the population,

(ii) glucose almost completely inhibits constitutive beta-galactosidase synthesis by cells grown in the presence of amino acids,

(iii) glucose almost completely inhibits constitutive beta-galactosidase synthesis in cells grown in the absence of amino acids,

(iv) the synthesis of induced enzyme is inhibited in cells grown in the presence of amino acids, and

(v) there is only a partial inhibition of induced enzyme formation in cells grown in the absence of amino acids.

Thus, the effect of glucose on beta-galactosidase synthesis in S. fragilis appears to be opposite to that reported in E. coli.

The presence of glucose enhances the growth rate of S. fragilis. In experiments reported above, where the growth rate was increased, there was an apparent inhibition of beta-galactosidase synthesis in E vs M graphs. It is, therefore, pertinent to compare dE/dM at given generation times in

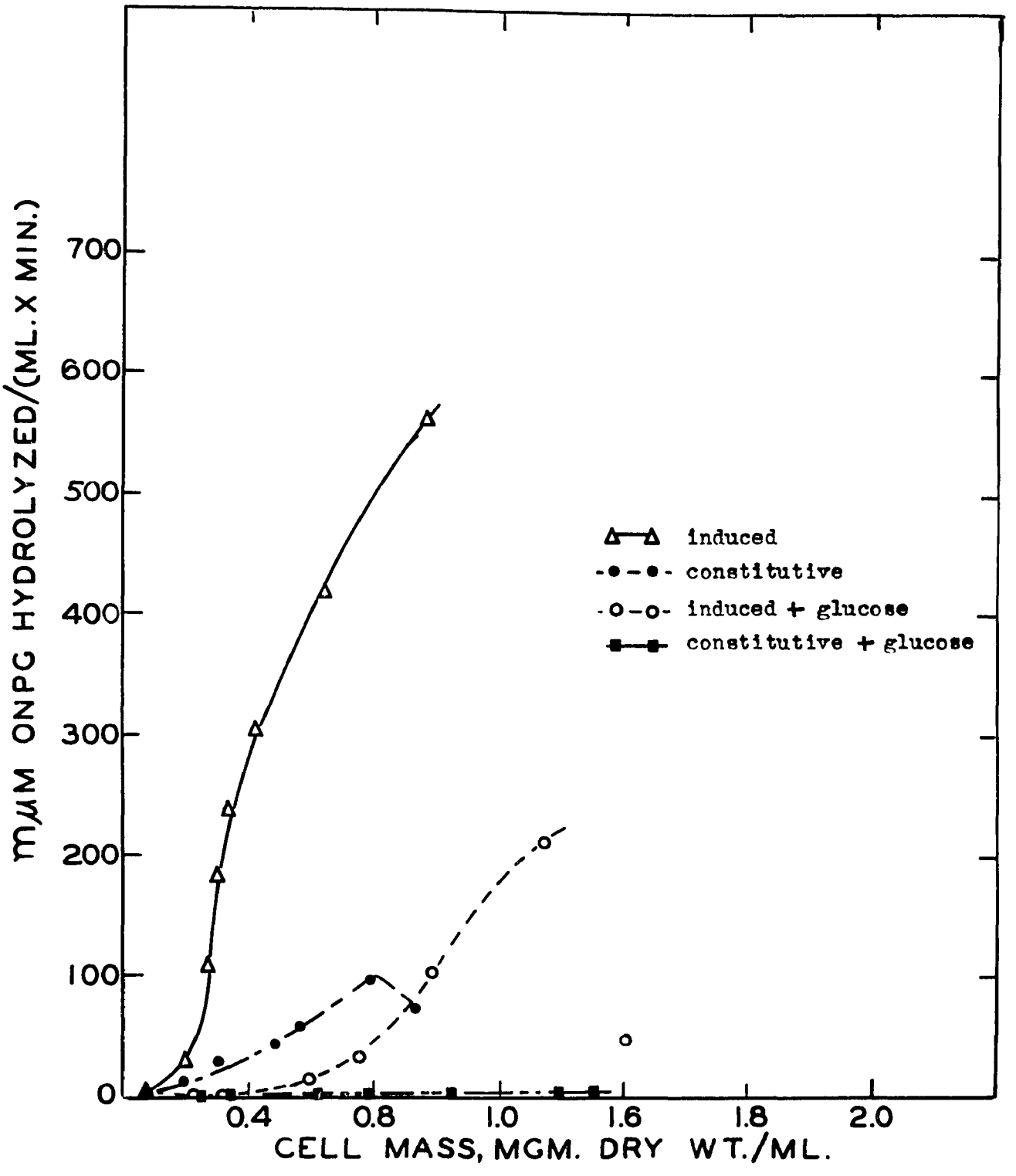


Fig. 18. Effect of 3% glucose on induced and constitutive beta-galactosidase synthesis.

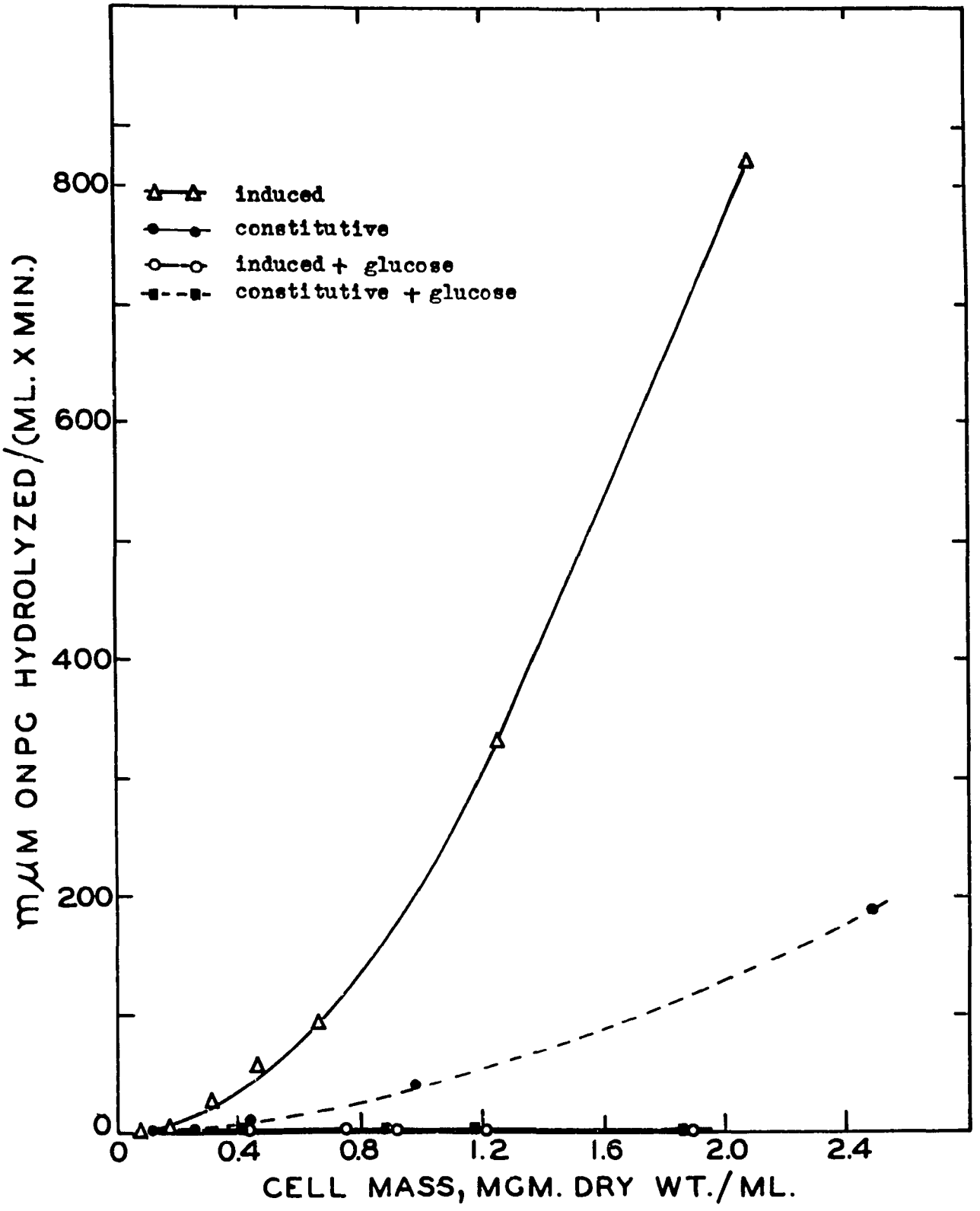


Fig. 19. Effect of 3% glucose on induced and constitutive beta-galactosidase synthesis in the presence of A.A.

control and glucose cultures. As seen in Table 15, the values of dE/dM are lower in glucose than in the corresponding control at given values of g. This result is clearly exemplified by induced enzyme synthesis. In the presence of amino acids, dE/dM equals 89 at a g of 2.2 when glucose is absent, whereas dE/dM equals 0.33 at a g of 2.3 when glucose is present. In the absence of amino acids, dE/dM equals 82 at a g of 6.8 when glucose is absent, whereas dE/dM equals 9.1 at a g of 8.7 when glucose is present.

The reason for the initial decrease in total enzyme in the population is not known. However, it is known that cells grown in Davies medium with 1 % ethanol must be induced to glucose utilization under anaerobic conditions. Also, in 1 % glucose, there is no drop of enzyme activity at the beginning of the experiment. Therefore, 2 % glucose added to cells grown in 1 % ethanol plus 1 % glucose may cause an additional induction for glucose with a resulting decrease in the level of beta-galactosidase.

This hypothesis is supported by reports of induction to one enzyme effecting a decrease of the enzymes or proteins already present in the cell. Spiegelman and Dunn (46) have noted that induction to galactose causes a decrease in maltase concentration of maltase induced cells. Furthermore, there is a decrease in the level of Pz with induction to beta-galactosidase in E. coli (47,48).

Ben Ishai and Spiegelman (49) have shown that in the presence of glucose, amino acids can more readily enter the yeast cell and cause a reduction of the nucleotide pool. Concomitant with this phenomenon, there is an inhibition of enzyme synthesis, which can be relieved by the addition

TABLE 15

RELATIONSHIP BETWEEN dE/dM AND GENERATION TIME FOR INDUCED AND CONSTITUTIVE BETA-GALACTOSIDASE SYNTHESIS IN THE PRESENCE AND ABSENCE OF GLUCOSE

Gal.	A.A.	Glu.	dE/dM	g	dE/dM	g	dE/dM	g	dE/dM	g	dE/dM	g
-	-	-	89	14.5	63.5	16.5	56.5	13.7	49.6	9.4	44.2	8.9
-	+	-	127	19.2	9	2.7	7.7	2.0	9.3	2.9	47.5	7.8
+	-	-	102	9.3	82	6.8	484	26.4	700	36.2	605	33
+	+	-	75.5	8	19.6	2	43	2	89	2.2	101	2.4
-	-	+	1.1	5.1	2.2	9.9	2.7	12.2	.91	5.7	0	15.1
-	+	+	1.1	1.6	0.1	2.2	.14	4.4	0	14		
+	-	+	2.5	5.6	9.1	8.7	19.2	12.7	57	15	121	16
+	+	+	.34	1.6	.32	1.5	.33	2.3	.46	2.9	1.1	4.5

These calculations are made from the data in figures 16 and 17.

Only positive values of dE/dM are noted.

of nucleosides. For this reason, experiments similar to those described above have been performed with the addition of a mixture of nucleosides, each at a level of 100 micrograms per ml. The glucose inhibition was not relieved; indeed the growth rate was slightly increased, resulting in a comparable decrease in the value of dE/dM.

C. Beta-galactosidase Synthesis in Resting Cell Suspensions

There was a two-fold purpose in performing experiments with resting cell suspensions. Not only was it of interest to see if this enzyme could be synthesized by resting cells, but also the kinetics of the induction process should be considerably simplified in the absence of growth.

1. Experimental methods

Before resting cell experiments could be performed, it was important to know the type of cell and the composition of a suspending medium which would be optimum for enzyme synthesis. Accordingly, cultures of S. fragilis, strain Y 665, were grown in Davies medium, 1 % in ethanol, with and without the addition of 0.25 % amino acids. The cells were harvested at optical densities of 0.5 and 1.05-1.1, washed twice in cold double distilled water, and resuspended to an O.D. of 1.2. Davies medium and 0.1 M potassium phosphate buffer of pH 6.8, each supplemented with 1 % ethanol, were employed as suspending media. 0.01 M galactose and 0.25 % amino acids were added to each suspending media such that induced and constitutive enzyme formation were studied in the presence and absence of amino acids in each medium. 15 ml of cell suspension was dispensed into a 20 x 2.5 tube, the additions made, and the tube incubated with shaking as previously described. At regular intervals, O.D. measurements were made, and simultaneously samples were removed for enzyme assay.

2. Experimental results

As compared to the synthesis of induced maltase in S. cerevisiae, strain K (46) relatively little beta-galactosidase was synthesized under any of the conditions tested. The results are summarized below.

(i) In the absence of relatively large increments of O.D., little enzyme was synthesized, unless the cells were incubated for 4 to 5 hours.

(ii) Over similar increments of mass, E/E_o was smaller than that found in the previously described growth experiments.

(iii) At a given g, dE/dM was smaller than in growing cell experiments previously described.

(iv) The quantity d log (E/M)/dt was greatest when young amino acid grown cells were suspended in Davies medium supplemented with amino acids. These conditions were followed in some of the experiments in the following section. Because the enzyme synthesis was small, experiments of this type were discontinued.

D. The Ability of Various Compounds to Induce Beta-galactosidase in S. fragilis

Many theories concerning the mechanism of inducer action have been advanced, and precise interpretation of these theories allow them to be tested experimentally. Monod and Cohn (50) have grouped such theories into the following two classes:

(i) The functional hypothesis - the synthesis of an enzyme is associated with activity of that enzyme. One interpretation of this hypothesis is that all inducers are substrates.

(ii) The equilibrium hypothesis - a reaction subject to the Mass Action Law, is conceived to occur between enzyme and inducer to form an enzyme precursor type of molecule. In the presence of inducer, the reaction is pulled in the direction of new enzyme formation. Again, one interpretation of this hypothesis would be that inducers are substrates.

It is because of these hypotheses that studies on the relation between the inductive capacity and substrate competence of inducers or substrates for an enzyme are of importance. Investigations of this nature have been performed with the maltase system in yeast (51,52), and the beta-galactosidase system in E. coli (10,53). Results of these studies amply demonstrate that:

(i) inducers are not necessarily substrates of the induced enzyme, and

(ii) complexants with the enzyme are not necessarily inducers.

However, it was deemed worthwhile to perform a similar investigation on the beta-galactosidase of S. fragilis, and to compare the results of such an investigation with those of the beta-galactosidase in E. coli.

Accordingly, a variety of compounds were tested for inductive capacity both under conditions of growth and under the conditions of slow growth described above in section C. Both types of experiments were performed with amino acid grown cells suspended in the Davies medium supplemented with 1 % ethanol, 0.01 M inducer, and 0.25 % amino acids.

Table 16 lists the compounds tested and whether or not they would induce. From E vs M graphs and dE/dM at a given generation time, methyl-beta-D-galactoside has proven to be the best inducer. n-butyl-beta-D-galactoside

TABLE 16

INDUCTIVE CAPACITY OF VARIOUS GLYCOSIDES FOR THE BETA-GALACTOSIDASE OF S. FRAGILIS, STRAIN Y 665

Glycoside	Inductive Capacity
Galactose	+
Beta-D-galactosides	
n-butyl	+
methyl	+
phenyl	+
ortho-nitrophenyl (ONPG)	+
4-glucose (lactose)	+
Alpha-D-galactosides	
methyl	-
phenyl	-
6-glucose (melibiose)	-
Beta-D-thiogalactosides	
phenyl	-
Arabinose	-
Beta-D-methyl arabinoside	-
Beta-L-methyl arabinoside	-

and phenyl-beta-D-galactoside are equally effective, followed closely by galactose. Lactose has the least inductive capacity. However, glucose released from the hydrolyzed lactose would be expected to partially inhibit enzyme synthesis under the conditions employed.

It should be noted that the order of inducer efficacy outlined above may not be real, for experiments were performed only at inducer levels of 0.01 M. However, a valid comparison between these compounds is difficult to make in view of the fact that all compounds which are inducers are also substrates, and furthermore their rates of utilization by the cells are different.

Kinetic evidence indicates that the mechanism of induction is the same for all inducers. The kinetics of induction are similar in the following respects:

(i) at the inducer level employed, for each g there is a unique dE/dM for each inducer,

(ii) after the exponential growth phase, enzyme is synthesized exponentially with respect to mass, and

(iii) the slope of the region of exponential enzyme synthesis is the same for all inducers.

Since constitutive and induced beta-galactosidase are identical by all tests employed, and are being studied in the same cell, it is impossible to evaluate the effect of inducers on constitutive enzyme formation. The compounds which are not inducers (see Table 16), have been found to have no effect on the synthesis of either constitutive or induced enzyme.

These data clearly show some differences in the specificities in the induction of the beta-galactosidases in E. coli (10) and S. fragilis. Alpha-galactosides are inducers for the enzyme in E. coli, and do not induce

the enzyme in S. fragilis. On the other hand, phenyl-β-D-galactoside is an effective inducer for the yeast enzyme, and is ineffective in E. coli. Phenyl-beta-D-thiogalactoside, which inhibits induction of the E. coli enzyme, has no effect on the induction of the yeast enzyme. Also, the bacterial system is induced by non-utilizable compounds, whereas all inducers for the yeast enzyme are also utilized by the cell.

E. Induction of Beta-galactosidase and Alpha-glucosidase in the Hybrid, FPR-1

The synthesis of beta-galactosidase and alpha-glucosidase in the hybrid FPR-1 was studied in order to attest the generality of the kinetics found for induced and constitutive beta-galactosidase synthesis in S. fragilis, strain Y 665.

Experiments to study enzyme formation in growing cells were performed as previously described for S. fragilis, Y 665. The Davies medium with 1 % ethanol was supplemented with 0.25 % amino acids, 0.03 M galactose, and 0.5 % maltose or alpha-methyl-glucoside, such that induced and constitutive enzyme formation of each of the enzymes was studied in the presence and absence of amino acids. Beta-galactosidase activity was measured by the ONPG method, alpha-glucosidase by the use of alpha-phenyl-D-glucoside.

The kinetics of formation of each of the enzymes was similar to that for the beta-galactosidase in strain Y 665. dE/dM varied with g, and after the exponential growth phase, both enzymes were synthesized exponentially with respect to mass. Thus these kinetics are not unique to the beta-galactosidase, nor to S. fragilis.

A note can be made concerning the relative inductive capacities of maltose and alpha-methyl-glucoside for the alpha-glucosidase in the hybrid,

FPR-1, and S. cervisae. In the former organism, alpha-methyl-glucoside is a more effective inducer than maltose. The situation is reversed in S. cervisae, strain K(28).

IV. DISCUSSION

As stated in the Introduction, the two primary aims of this Thesis are a determination of the properties of the induced and constitutive beta-galactosidases of S. fragilis, and a comparison of the kinetics of their synthesis. This Thesis is also concerned with manifestations of inducer activity and a comparison of beta-galactosidase properties and induction in E. coli and S. fragilis.

A. Number and Properties of beta-galactosidases in S. fragilis

For studies of this nature, it is requisite to know not only the properties but also the number of beta-galactosidases in the induced and constitutive cells.

It appears that the induced cell and the non-induced cell each possesses one stable beta-galactosidase respectively. The bases for this statement are summarized below.

(i) When crude cell-free extracts of induced or non-induced cells are subjected to starch column ionophoresis, there is only one peak of beta-galactosidase activity.

(ii) Both the beta-galactosidase in crude cell-free extracts and that purified by starch column ionophoresis hydrolyze similar compounds, i.e., lactose, ONPG, phenyl-beta-D-galactoside, n-butyl-beta-D-galactoside, and methyl-beta-D-galactoside.

These methods do not eliminate the possible presence of beta-galactosidases too unstable to withstand starch column ionophoresis. However, such unstable

enzymes cannot account for any quantitatively significant portion of the hydrolytic activity on lactose, ONPG, or methyl-beta-D-galactoside because:

(i) activities on these three substrates are stable at 5°C for 48 hours or longer, and

(ii) ONPG hydrolytic activity is recovered quantitatively from the starch column.

Therefore, the use of these compounds as substrates allows measurement of beta-galactosidase activity attributable to one molecular species.

In order to determine if the beta-galactosidase in induced cells was the same as that in non-induced cells, the two enzymes were compared with respect to: ionic requirements, pH optima, response to stabilizing agents, mobilities, and substrate specificities. Since the beta-galactosidases in the genetically alike induced and constitutive cells are identical by all of these tests, it appears that they are the same enzyme. This conclusion invalidates the hypothesis that the difference between constitutive and induced beta-galactosidase formation can be attributed to the synthesis of different enzymes.

In their investigations on this problem, Monod and his coworkers (50) demonstrated that the beta-galactosidase of an inducible E. coli was identical to that of its constitutive mutant. In addition, Pollock et al (54) have shown penicillinase in constitutive B. cereus to be identical to the enzyme in induced cells.

B. Kinetics of Beta-galactosidase Synthesis in S. fragilis

1. Introduction

From the above discussion, it is clear that the beta-galactosidase system in S. fragilis is well suited for a comparison of the kinetics of

induced and non-induced enzyme syntheses. The comparison is based upon the suggestion that if the kinetics of synthesis of induced and constitutive enzymes are qualitatively similar, the same basic mechanisms are involved in the formation of both enzymes. Qualitatively similar kinetics are exhibited for the synthesis of beta-galactosidase by an inducible strain of E. coli and a constitutive mutant of it (45,55).

In this kinetic study, comparisons are also made with the following two types of plots reported for induced enzyme formation.

(i) There is a linear relationship between log of enzyme activity and time when resting cell suspensions of yeast are induced for alpha-glucosidase (56).

(ii) There is a linear relationship between increase of beta-galactosidase and increments of cell mass with exponentially growing E. coli (55).

The first type of kinetics indicates an autocatalytic reaction. It is therefore of great importance to determine if the proportionality between rate of increase of enzyme and enzyme concentration predicates a direct causal relationship between these two quantities. Two other conditions which could result in these kinetics have been ruled out by Spiegelman et al (57) and by Benzer (58). We may therefore conclude that the first order kinetics do result from an autocatalytic mechanism.

The second type of plot (i.e. Relative Plot or E vs M) gives information on the proportion of the overall protein synthesis which is diverted into enzyme synthesis. The linear relationship which is observed during exponential growth of E. coli shows that under this growth condition, a constant proportion of new protein is beta-galactosidase.

The unique feature of the Relative Plot is that the factor of time

has been eliminated, and comparison of enzyme formation in fast and slow growing cultures is facilitated. However, the time parameter should not be neglected, for the relationship $dE/dM = k$ is equivalent to $\frac{dE/dt}{dM/dt} = k$. Considered on the per cell basis, dM/dt is inversely proportional to the generation time. It is evident that two cells synthesizing enzyme at the same rate would exhibit different values of k if their generation times differed. It is one of the purposes of this Thesis to examine the relationship between dE/dM and generation time (g). Subsequent to this work, Rickenberg and Lester (59) have shown the relative rate of beta-galactosidase synthesis in resting E. coli suspensions to be greater than that of exponentially growing cells, and Wainwright and Nevill (60) note an "inverse correlation between the total amount of enzyme formed and the extent of increase in cell mass after addition of nitrate inducer" in E. coli.

2. Kinetics observed for beta-galactosidase synthesis in S. fragilis

The data presented herein clearly demonstrate the existence of two kinetically distinct regions of beta-galactosidase synthesis in growing cultures of induced and non-induced S. fragilis. These two regions are described as follows:

(i) Region 1

$E = kM - b$ 1a

or $dE/dM = k$ 1b

(ii) Region 2

$\log E = cM + f$ 2a

or $dE/dM = cE$ 2b

where E is $m\mu M$ ONPG hydrolyzed/(ml x min) and M is mgm dry wt of cell material per ml.

a. Region 1. The direct proportionality between E and M occurs during exponential growth, i.e.,

$$dM/dt = jM \qquad 3$$

where t is time in hours.

If equations 1 and 3 are combined,

$$dE/dt = jE + jb \qquad 4$$

According to equation 4, the rate of enzyme synthesis is a function of the enzyme concentration present, the growth rate j, and b. If the term jb is negligible, then the synthesis in this region follows first order. The importance of jb can be determined on the following premises:

(i) If jb is negligible, a linear relationship should be observed between log E and t.

(ii) If there is a linear relationship between log E and t, the slope of the graph log E vs t should be the same as that of the plot log M vs t.

An apparently linear relationship was observed in plots of log E vs t. However, the slope of this plot was not the same as log M vs t. Therefore jb is not negligible, and the synthesis of enzyme in this region cannot be regarded as a simple autocatalysis.

The ratio of newly formed mass which is new enzyme is given by k in equation 1b. This result simulates that found by Monod (55) for beta-galactosidase synthesis in E. coli.

If there is an interaction between rate of enzyme synthesis and rate of mass synthesis, k will be affected by the generation time, g. The existence of such an interaction is demonstrated by the following experimental results:

(i) Figures 13 and 15 show that dE/dM increases with g in a given growth tube.

(ii) In constitutive enzyme synthesis, dE/dM at a value of

g occurring during the initial lag in growth is equal to the dE/dM found later at the same g.

(iii) Experiments performed in the absence of amino acids show the same dE/dM at recurring values of g.

(iv) Figure 10 shows an E vs M plot of experiments performed with and without amino acids. At comparable cell densities, the amino acid grown culture has the lower g and a lower dE/dM.

(v) Similarly, Figure 14 depicts an E vs M plot of experiments performed in the presence of 0.5 % ethanol and 1 % ethanol. At comparable cell densities, the 0.5 % ethanol grown culture has the lower g, and lower dE/dM.

(vi) A linear relationship does not exist between E and t.

These results, and those of Rickenberg and Lester (59) and Wainwright and Nevill (60) clearly demonstrate that an interaction exists between the rate of mass synthesis and the rate of enzyme formation. Therefore, differences of the k in Relative Plots of fast and slow growing cultures may be caused not by the direct influence of a test substance on the rate of enzyme formation, but by differences in generation times of the cultures. The plot dE/dM vs g is advantageous because comparisons at the same g eliminate the effect of the test substance on g.

b. Region 2. Equation 2b states that the increment of enzyme per unit increase in mass is dependent upon the concentration of enzyme already present in the culture. This region occurs sometime after the exponential phase of growth, and because of the nature of the equation, the ratio dE/dM should no longer be strictly dependent upon g.

The kinetics observed during this region simulate those reported by Spiegelman (56) for induced enzyme formation in resting cell suspensions of yeast. The Spiegelman system obeys the equation $dE/dt = rE$, whereas the kinetics observed in region 2 are described by the equation $dE/dM = cE$, where E is enzyme activity, t is time, M is cell mass, and r and c are constants.

3. Comparison of kinetics of induced and constitutive enzyme syntheses

The fact that the beta-galactosidases of induced and constitutive cells appear to be identical provokes the hypothesis that the limiting factors are the same for each. This hypothesis is supported by the following kinetic similarities.

(i) Region 1 is found with both types of cells during exponential growth.

(ii) Before region 2 occurs in the history of the culture, dE/dM varies with g in both types of cells.

(iii) Region 2 is found with both types of cells sometime following the exponential growth phase.

(iv) The slopes of region 2 are the same in the presence of amino acids.

(v) In Relative Plots, there is an apparent inhibition of both types of enzyme synthesis by the addition of amino acids, or by a reduction in ethanol concentration. This inhibition is attributable to decreases in g with resulting lower dE/dM values.

The kinetics of beta-galactosidase synthesis in induced and constitutive cells differ quantitatively in the values of k in equation 1a. The lower value is observed during constitutive synthesis.

4. Interpretation of kinetics

The rate of mass synthesis appears to influence the rate of beta-galactosidase synthesis; in constitutive enzyme formation, when g varies from x to y to x, where $x > y$, dE/dM varies from a to b to a, where $a > b$. These results indicate a competition between mass and enzyme syntheses for a common material or materials, X. Thus, region 1 would be the result of mass synthesis causing a limitation of X for enzyme synthesis. When mass synthesis is limited by some factor other than X, the concentration of X should increase until it is no longer limiting for the synthesis of E. Apparently this situation prevails during region 2: g increases, and enzyme synthesis becomes first order in E. Autocatalytic kinetics also occur, as would be expected by this interpretation, in resting cell suspensions of yeast (56).

Since qualitatively similar kinetics were found herein for alpha-glucosidase synthesis, the above interpretation can be generalized. It therefore appears that the same general pathway is employed in the syntheses of beta-galactosidase and alpha-glucosidase.

Attempts to define X by additions of amino acids, ribose, nucleosides, and purines and pyrimidines have been unsuccessful.

5. Postulated mechanisms of autocatalytic kinetics

Since a rate-limiting autocatalytic step is apparently involved in enzyme synthesis, the elucidation of its mechanism is of importance. The facts to be considered in any postulated scheme for such a mechanism are given below.

(i) A cytoplasmic particulate unit which is heritable in the presence of inducer appears to be involved in enzyme formation (61,62).

(ii) The unit probably exists in two forms, active and inactive (63). The existence of a third form, easily activated, also has been postulated (64). However, the data upon which evidence for the third form is based could also be attributable to an increased rate of inducer uptake with induction as described by Cohen for E. coli (65), and seen herein for S. fragilis.

(iii) The active particles are short-lived, reverting either to the inactive form or being destroyed (43).

(iv) The enzyme is probably not the enzyme forming site (10).

(v) There is no enzyme synthesis in the absence of a net increase in RNA (66).

Spiegelman and Campbell (64) have recently forwarded an hypothesis based upon the following assumptions:

(i) the enzyme forming site is distinct from E,

(ii) the enzyme forming site exists in three interconvertible forms; inactive, or EFS; readily activated, or EFS.E, where E is enzyme; and active, or EFS.E.I, where I is inducer,

(iii) the total number of enzyme forming sites per cell is constant, but the ratios of the different forms may be changed under the influence of the inducer and enzyme, and

(iv) to make enzyme, EFS must be activated by both E and I.

Autocatalysis is considered to be the result of a feed-back mechanism wherein enzyme produced is employed to activate EFS.

Because of the paucity of data, schemes based upon the following assumptions could also be operative:

(i) a particulate element for enzyme synthesis exists in two forms, active and inactive,

(ii) the particle is RNA or RNA-protein complex, and serves as a template for enzyme formation,

(iii) for every enzyme molecule synthesized, one template is irreversibly destroyed,

(iv) the total number of templates remains constant by virtue of the synthesis of one new template for every template irreversibly destroyed.

A scheme is diagrammed below:

$$\text{P-P} \xrightarrow[1]{} \text{EFS*} \xrightarrow[2]{\text{I*}} \text{EFS*.I*} \xrightarrow[3]{\text{AA}} \text{EFS} + \text{E} + \text{I*},$$

where P-P are purines and pyrimidines, EFS* is inactive enzyme forming site, I* is inducer, EFS*.I* is the activated particle, AA are amino acids, EFS is the irreversibly inactivated residue, and E is enzyme. The rate-limiting autocatalytic step can be considered to occur at 1, 2, or 3. In this diagram, the catalyst must be a product appearing to the right of whichever arrow represents the slow step.

The autocatalytic step could also be caused by the degradation of an inhibitor of enzyme synthesis. The following assumptions could be made:

(i) the released inhibitor, or degraded inhibitor catalyzes inactivation of the inhibitor,

(ii) one enzyme molecule is synthesized with the inactivation of each inhibitor molecule,

(iii) there is a large supply of enzyme forming site-inhibitor complex,

(iv) enzyme forming site is RNA or RNA-protein complex, and each enzyme forming site can synthesize only one enzyme molecule.

A scheme is outlined below:

$$P\text{-}P \xrightarrow[1]{} EFS^*.Inh \xrightarrow[2]{I^*} EFS^*.I^* + Inh \xrightarrow[3]{AA} EFS + E + I^*,$$

where Inh is the inhibitor moiety. Autocatalysis can be achieved if Inh catalyzes step 1 or 2.

The above mechanisms are applicable to induced enzyme synthesis. It is pertinent to apply them to constitutive enzyme formation because of the kinetic similarities in induced and constitutive enzyme syntheses described herein. When this is done, it is seen that either the constitutive cell must synthesize its own inducer, or the inducible cell must contain an inhibitor of enzyme synthesis which can be inactivated by the inducer. The former alternative is supported by the phenomenon of sequential adaptation (67) and by the studies of Vogel and Davis (68) on n-acetyl-ornithinase. Tests for an inhibitor of enzyme synthesis which is unique to the inducible cell have been unsuccessful, although an inhibitor of enzyme synthesis has been found in both inducible and constitutive cells (43).

C. Function of Inducer

Some information on the function of the inducer may be gained by studying its effects on the cell. For this reason, the manifestations of inducer action are listed below.

1. Increased enzyme activity

The beta-galactosidases of induced and constitutive cells are the same. Therefore, addition of inducer increases the rate of synthesis of an

enzyme already formed by the cell. In so doing, the inducer must effect an increase in the amount of X available for enzyme synthesis during Region 1, if the kinetic interpretation is correct.

2. Crypticity changes

It is clear from the data herein presented that the inducer also causes a change in the crypticity of the cell. The crypticity factor, defined as the ratio of rate of hydrolysis on a specified substrate by extract to rate of hydrolysis on the same substrate by intact cells, decreases on induction. Furthermore, the percentage decrease was not the same for all substrates.

From the discussion presented in Section IV A, these crypticity changes cannot be attributed to the elaboration of more than one enzyme by the induced cell, or to differences in the beta-galactosidases from induced and constitutive cells. It is therefore possible that the rate at which substrate and enzyme are brought together can be varied. This postulate is supported by data on the beta-galactosidase in E. coli. The rate of uptake of sulfur labelled methyl-beta-D-thiogalactoside by E. coli increases with time of exposure to beta-galactosidase inducers (65), and the rate-limiting step appears to be an inducible beta-galactoside transfer enzyme (69). The presence of an inducible beta-galactoside transfer enzyme in S. fragilis could result in crypticity changes of the type reported herein. However, if this hypothesis is correct, an increase in the postulated transfer enzyme would not necessitate a decrease in crypticity factor as found in S. fragilis. In E. coli, the beta-galactosidase crypticity factor is increased by induction (70).

On the assumption that there is an inducible beta-galactoside transfer enzyme in S. fragilis, the data presented in Table 12 indicate its

specificity. These data demonstrate that induced cells utilize galactosides at a greater rate than free glucose and galactose, whereas the reverse situation holds for non-induced cells. If free glucose and galactose, and the hydrolysis products of galactosides, are utilized by the same pathways, it follows that the transfer enzyme is more specific for galactosides than for their constitutent hexoses. Furthermore, this interpretation dispenses with the elusive series of enzymes postulated for the direct utilization of lactose (15,16,17,18,19).

3. Comparison of specificities of beta-galactosidase and beta-galactosidase enzyme forming site

Because one interpretation of both the Functional and Equilibrium hypotheses of inducer action is that substrates are inducers, the molecular configurations of inducers and substrates should be compared. From the data presented in Tables 7, 8, and 16, it is clear that to be an inducer or substrate, a compound must 1) have an intact galactosidic ring, 2) have the substituent group linked to the C_1 carbon of the galactosidic ring through an oxygen, and 3) have the beta configuration. Excepting galactose, among the compounds tested, all inducers are also substrates, and all substrates are also inducers. It would therefore appear that the specificities of the enzyme and the enzyme forming site are identical. However, the studies on the beta-galactosidase of E. coli (10) and the alpha-glucosidase of yeast (52) have shown that the specificities need not be the same. Compounds which are non-substrate inducers have been documented for both systems. In addition, a non-inducer enzyme complexant has been described for the former system.

4. Effect of inducer concentration on induced enzyme synthesis

Figures 8a, 8b and 11 demonstrated a concentration-dependent inhibition of induced enzyme synthesis at high levels of inducer. Landman (71) has reported a similar inhibition of the beta-galactosidase of

Neurospora crassa, and has hypothesized the inhibition of a two point attachment of the inducer to the enzyme forming site. An alternative hypothesis is that galactose must be converted by the cell into active inducer, I*, and unconverted galactose can compete with I* for the enzyme forming site. Both of these hypotheses assume a dependence of the cell's galactose concentration upon the galactose concentration in the surrounding medium. This assumption is supported by the findings of Cohen and Monod (65,70).

D. Effect of Glucose on Beta-galactosidase Synthesis

It has been demonstrated that glucose affects beta-galactosidase synthesis in S. fragilis as follows:

(i) in the presence or absence of amino acids, glucose inhibits constitutive enzyme formation,

(ii) in the presence of amino acids, glucose inhibits induced enzyme synthesis, and

(iii) in the absence of amino acids, glucose partially inhibits induced enzyme formation.

These results are of interest, because they permit both a partial separation in the conditions of constitutive and induced enzyme formation, and a demonstration of enzyme induction in the presence of glucose. It should be noted that the effect of glucose is greatest when the rate of beta-galactosidase synthesis is lowest. Furthermore, the amino acid effect in (iii) above is not solely attributable to an increased growth rate. It could also be caused by an induction to amino acid utilization resulting from the increased rate of uptake of amino acids in the presence of glucose (49).

These results should be compared to those of E. coli. In the bacterial cell, glucose affects beta-galactosidase synthesis in the following

manner:

(i) induced enzyme synthesis is inhibited when inducer and glucose are added simultaneously (44),

(ii) induced enzyme synthesis is not inhibited in fully induced cells in the presence of inducer (44),

(iii) constitutive enzyme synthesis in the constitutive mutant is not inhibited (45).

Here again, the system which makes enzyme at the lowest rate is inhibited by glucose.

Since both the E. coli and S. fragilis experiments were performed under conditions which could allow for glucose induction, the results are most easily interpretable on the following assumptions:

(i) the constitutive cell synthesizes an inducer, whereas the inducible cell does not,

(ii) there is a competition by mass synthesis, glucose induction, beta-galactosidase induction, and amino acid induction for the postulated material X, and

(iii) the level of X diverted to induced enzyme synthesis is dependent upon the level of the inducer.

It is clear that since induction can be initiated in the presence of glucose in S. fragilis, glucose cannot inhibit induced enzyme synthesis by preventing the entrance of inducer into the cell (cited in 41).

E. Comparison of Properties and Induction of Beta-galactosidases in E. coli and S. fragilis

When considering the ontogeny of induced enzymes, two queries are immediately provoked:

(i) are enzymes with similar enzymatic activities identical in all microorganisms, and

(ii) when superficially alike enzymes in two different microorganisms are induced, are the specificities and conditions for induction identical?

In their studies on the first of these questions, Monod and Cohn (50) have found that the beta-galactosidases of widely diverse microorganisms are different. Thus, the beta-galactosidases in E. coli and S. fragilis differ immunologically. The data presented herein allow further elaboration of their similarities and differences.

The bacterial (72) and yeast enzymes are unlike in their response to various stabilizing agents. The activity of the bacterial enzyme is unaffected by cysteine, whereas the activity of the yeast enzyme is stabilized and may be increased by additions of cysteine, glutathione, alanine, or methionine.

There are both similarities and dissimilarities in their response to ions. Both enzymes are insensitive to inorganic phosphate, both enzymes require either potassium or sodium ion, sodium being the superior activator for ONPG hydrolysis. However, they are different in their response to divalent ions. Whereas the beta-galactosidase from yeast is activated by magnesium ion and manganese ion, and slightly activated by calcium ion, the activity of E. coli enzyme is unaffected by magnesium ion, inhibited 62 % by 0.02 M $MnCl_2$, and inhibited 88 % by 0.1 M $CaCl_2$ (72).

Both the S. fragilis and the E. coli (10) enzyme hydrolyze methyl-beta-D-, n-butyl-beta-D-, phenyl-beta-D-, ortho-nitrophenyl-beta-D-, and glucose-beta-D-galactosides. Neither enzyme hydrolyzes phenyl-beta-D-thiogalactoside, methyl-beta-D-thiogalactoside, or melibiose. However, the enzymes differ in their relative specificities: the ratio of rate of ONPG

hydrolysis to rate of lactose hydrolysis approximates 1 for the yeast enz[redacted],
and 3 for the E. coli enzyme (72).

On comparing the specificities of the induced enzyme forming sy[redacted]m,
it is seen that the bacterial enzyme is induced by melibiose, methyl-alph[redacted]
D-galactoside, and methyl-beta-D-thiogalactoside (10). None of these
compounds are inducers for the yeast system. Furthermore, phenyl-beta-D-
thiogalactoside inhibits induced enzyme formation in E. coli (10), but no[redacted]
in yeast.

V. SUMMARY

An investigation has been performed on the properties and kinetics of syntheses of the beta-galactosidases in induced and constitutive S. fragilis, and on the specificity of the induction. The results are summarized below.

1. As shown by starch column ionophoresis and substrate specificity, there appears to be one stable beta-galactosidase in induced and constitutive cells of S. fragilis.
2. The beta-galactosidases in induced and constitutive S. fragilis are indistinguishable on the basis of environmental effects on activity, mobility, and substrate specificity.
3. All beta-D-galactosides tested (excluding beta-D-thiogalactosides) are both inducers and substrates for the enzyme.
4. The crypticity of the enzyme in induced cells is less than that in non-induced cells, whereas in E. coli, the reverse situation has been described.
5. Under growth conditions, both constitutive and induced cells of S. fragilis and a hybrid of S. fragilis and Z. dobzhanskii exhibit two types of kinetics for beta-galactosidase and alpha-glucosidase biosyntheses. In exponential growth, the ratio of new enzyme formed to new mass synthesized is a constant ($E = kM - b$, or $dE/dM = k$, where E is enzyme, M is cell mass, and k and b are constants). At sometime after exponential growth, the ratio of new enzyme formed to new mass synthesized is proportional to the concentration of the enzyme, i.e., $\log E = cM + f$, or $dE/dM = cE$, where E is enzyme, M is mass, and c and f are constants.

6. When the kinetics of enzyme synthesis do not obey the equation log $E = cM + f$, the ratio of new enzyme formed to new mass synthesized, i.e., dE/dM, increases with increased generation time of the culture.
7. The same basic mechanism for induced and constitutive enzyme formation is indicated by the qualitative similarities in their kinetics of synthesis, and by the fact that the constants c in the equation log $E = cM + f$ are the same in the presence of amino acids.
8. Possible mechanisms for induced and constitutive enzyme formation are discussed.
9. The beta-galactosidases in E. coli and S. fragilis differ not only in their properties but also in the effects of compounds on their syntheses. The ionic requirements, antibody precipitation and substrate specificities are different. Also, the E. coli enzyme is induced by non-substrate beta-D-galactosides, methyl-beta-D-thiogalactoside, and alpha-D-galactosides, whereas the S. fragilis enzyme is not. Furthermore, glucose inhibits the initiation of induced E. coli beta-galactosidase, and has no effect on the constitutive enzyme formation. Glucose inhibits constitutive enzyme synthesis in S. fragilis, whereas induced enzyme formation can be initiated and maintained in its presence.

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